

PREVENTION OF CADMIUM INDUCED  
IMMUNOPATHOLOGY BY ZINC IN MICE

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BADRUL ALAM CHOWDHURY









# PREVENTION OF CADMIUM INDUCED IMMUNOPATHOLOGY BY ZINC IN MICE

BY

© Badrul Alam Chowdhury, M.B., B.S.

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Studies in partial fulfillment of the  
requirements of the degree of  
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## ABSTRACT

Cadmium is an ubiquitous toxic metal to which everyone is exposed at low levels. It is toxic to almost every organ system of the body including the immune system. In this study, the effects of a relatively low dose of cadmium on the immune system of mice and the effects of a moderately large dose of zinc on cadmium-induced immunopathology were studied. Six-week old C57BL/6 male mice were exposed to 50 ppm cadmium in drinking water for 3 weeks, and killed 0, 3 and 6 weeks after cessation of treatment. In some groups, 500 ppm zinc was added to the drinking water with or after cadmium treatment. The number of IgM and IgG antibody-forming cells in the spleen was higher in cadmium-treated mice as compared to non-treated controls at 0 week. Concurrent zinc administration prevented the enhancement of antibody-forming cell response. Proliferative response of spleen cells to the T cell mitogens phytohaemagglutinin and concanavalin A tended to be high in cadmium-treated mice and zinc administration after exposure to cadmium tended to lower it. The number of CD8<sup>+</sup> cells in the spleen was lower and the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> cells, reflecting the balance of immunoregulatory T lymphocytes, was higher in cadmium treated mice. Concurrent zinc administration prevented the alteration of T cell subsets. Suppressor cell activity tended to be low in cadmium treated mice. Natural-killer cell activity in the spleen was low in cadmium treated mice and concurrent zinc treatment prevented the suppression. B cell count in the spleen and antibody production by splenic lymphocytes on pokeweed mitogen

stimulation was not altered by cadmium or zinc treatment. Cadmium and zinc treatment had no effect on liver, kidneys, spleen and thymus weights and lymphocyte content of spleen, thymus and peripheral blood. Use of immunofluorescence with anti-mouse IgG and complement C3 showed no evidence of autoimmune reaction in kidney sections. On electron microscopic examination, ultrastructural alterations were seen in proximal tubular epithelial cells of the cadmium treated mice. Mitochondria were increased in number and the cisternae were distorted, and inside several cells electron dense materials were seen. Liver and kidney cadmium concentrations were high in cadmium-treated mice as compared to non-treated controls. Tissue cadmium levels were lower in mice treated with both zinc and cadmium than in those treated with cadmium alone. The results suggest that a relatively low dose of cadmium exposure produces immune alterations in mice that can be prevented by a moderately large dose of zinc.

INDEXING KEY WORDS : cadmium, zinc, cellular immunity

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## PUBLICATIONS

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2. Chowdhury BA, Chandra RK. Nutrition, Immunity and Resistance to Infection. In: Jeffrey Bland, ed. 1986 A Year in Nutritional Medicine. 2nd edn. pp.59-84, Keats Publishing, New Cannan, Connecticut, U.S.A., 1986.
3. Chowdhury BA, Chandra RK. Biological and Health Implications of Toxic Heavy Metal and Essential Trace Element Interactions. Prog Food Nutr Sci 1987; 11:57-113.
4. Chowdhury BA, Friel JK, Chandra RK. Cadmium-induced Immunopathology is Prevented by Zinc Administration in Mice. J Nutr 1987; 117:1788-1794.

5. Chowdhury BA, Friel JK, Chandra RK. Cadmium-induced Immunopathology is Prevented by Zinc Administration in Mice. Abstract. Proc Can Fed Biol Soc 1986; 29:103.
6. Chowdhury BA, Chandra RK. Alteration of Immune Functions in Cigarette Smokers is Corrected by Zinc Therapy. Abstract. J Am Col Nutr 1987; 6:443.
7. Chowdhury BA, Chandra RK. Metal Compounds and Immunotoxicology. Effects and Immunotoxicological Biomonitoring. In: Ernest Merian, ed. Metals and Their Compounds in the Environment. Occurrence, Analysis, and Biological Relevance. VCH Verlagsgesellschaft, Weinheim, Federal Republic of Germany. (Submitted, 1988)
8. Chowdhury BA, Chandra RK. Effect of Zinc Administration on Cadmium-induced Suppression of Natural-killer Cell Activity in Mice. (Submitted, 1988)



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## LIST OF ABBREVIATIONS

BSS	=	balanced salt solution
°C	=	Celsius
C	=	complement
Chl	=	cholesterol
Cd	=	cadmium
CD	=	cluster of differentiation
Cr	=	chromium
CO <sub>2</sub>	=	carbon-dioxide
Con A	=	concanavalin A
Cu	=	copper
Cys	=	cysteine
ELISA	=	enzyme-linked immunosorbent assay
FITC	=	fluorescein isothiocyanate
g	=	gram
HDL	=	high-density lipoprotein
Ig	=	immunoglobulin
K	=	kelvin
Kg	=	kilogram
LDL	=	low-density lipoprotein
mg	=	milligram
MHC	=	major histocompatibility complex
ng	=	nanogram
NK	=	natural-killer

PBS	=	phosphate-buffered saline
PBS-T	=	phosphate-buffered saline-Tween 20
PFC	=	plaque-forming cells
PHA	=	phytohaemagglutinin
RPMI	=	Rosewell Park Memorial Institute
SCA	=	suppressor cell activity
SRBC	=	sheep red blood cells
Wk	=	week
Zn	=	zinc

## CHAPTER 1

### INTRODUCTION

#### 1.1. TRACE ELEMENTS

The bulk of living matter consists of five elements, namely, carbon, hydrogen, nitrogen, oxygen and sulfur. They are present in tissues in concentrations of grams per kilogram and their adult human requirements are in the range of grams per day. The macrominerals sodium, potassium, calcium, magnesium, chlorine and phosphorus serve as structural components of tissues or as constituents of the body fluids. Their concentration in living tissue and adult human requirements are somewhat lower than the bulk elements, but still can be expressed as grams per kilogram and grams or fractions of a gram per day. The bulk elements and macrominerals are essential for the function of all cells and the organism as a whole. The remaining elements of the periodic table that occur naturally in the living tissues are present in much lower concentrations. Earlier analytical methods were unable to determine their precise concentration, hence they were often described as occurring in "traces" and the term "trace element" arose to denote them. Although present-day techniques can virtually estimate all elements in biological material with great accuracy, the term "trace element" is still retained in literature largely because it is hallowed by time and tradition. Nevertheless the term does provide a descriptive classification for a group of elements whose

tissue concentrations are very small and expressed in terms of milligrams or micrograms per kilogram (Mertz, 1981; Mertz, 1986).

Trace elements, which include all the naturally occurring elements except the bulk element and macrominerals, can be classified into two categories : first, those with proven essentiality; second, those for which proof of essentiality does not exist (Mertz, 1986). The essentiality of a trace element is judged by the criterion that its deficient intake consistently results in impairment of a function which is prevented or corrected by supplementation with physiological levels of the particular element and not by others. By this criterion chromium, cobalt, copper, fluorine, iodine, iron, manganese, molybdenum, selenium and zinc are accepted as essential at present, even though all of these do not present a practical nutritional problem to humans. In addition, arsenic, nickel, silicon, tin and vanadium are often included in the essential trace element group as deficiency symptoms have rarely been reported for them, but the site and mode of action of these elements are not yet clearly defined (Mertz, 1981; Mertz, 1986).

Most of the essential trace elements act primarily as structural components or catalysts in large molecules, hormones and enzymes. For example, iron is an essential component of the oxygen carrying protein haemoglobin, iodine is essential for functional activity of thyroid hormones, cobalt is the essential metal in vitamin B12, chromium acts as a cofactor for insulin, zinc is an essential cofactor in many metalloenzymes and copper is a key regulator of lysyl oxidase activity. Trace elements are required in an optimum level for their function. Deficiency will result in

suboptimal function, disease and in extreme cases death. Similarly, excess intake will cause toxicity and can have a fatal outcome. All essential trace elements thus have a bell-shaped dose response curve, the kurtosis of which varies. For some, like selenium, difference between optimum tissue level and toxic level is very narrow; while for others, like zinc, it is relatively wide. In the biological system optimum concentrations of trace elements are maintained by a series of regulatory mechanisms like absorption, distribution, metabolism and excretion. Some proteins are also involved in the metabolism of trace elements; like transferrin for iron, ceruloplasmin for copper, and metallothionein for heavy metals cadmium, zinc, copper and others. Although the function of all these proteins are not precisely known, most act as carriers or buffers against excess.

Among the second group of trace elements, for which no essential function has been described in man, some are of importance due to their proven toxicities. Lead, cadmium and mercury are important examples of this group. Although all trace elements, including the essential elements, have inherent properties of toxicity when present in excess; lead, cadmium and mercury serve no essential biological function, they are cumulative poisons and are toxic even at low doses. Their primary importance to biologists is due to their toxicity. Ideally none of these toxic metals should be present in the tissues, but due to industrial activity and human habits, all organisms are constantly exposed to these elements and accumulate a considerable amount of them in the tissues. Toxic heavy metals are brought up from the earth's crust for industrial use and usually very little of them are recycled. Thus their level in the environment is gradually increasing, so also are the levels in human and animal tissues.

(National Academy of Sciences, 1978). This is particularly true for lead and cadmium. The potential toxicities of low levels of these elements to which the general population is inadvertently exposed is thus of considerable public health importance.

Among many functions of trace elements, recent studies have also demonstrated their importance in the regulation of immune functions and host defense mechanisms. The impetus to the study of trace elements and immunity was generated from studies demonstrating consistent impairment of immune functions in children suffering from gross undernutrition, like protein-energy malnutrition, in which not only supply of protein and calories are deficient, but also body stores of most vitamins and essential trace elements are less than adequate (Chandra, 1972; Chowdhury and Chandra, 1986a; Chandra 1988).

The topic of trace elements and immunity has been the subject of several workshops, reviews and monographs (Chandra and Dayton, 1982; Beisel, 1982; Chowdhury and Chandra, 1986b; Chandra, 1987). The essential trace elements, particularly zinc, iron, copper and selenium are now established critical factors in the generation, maintenance and amplification of immune response. Deficiencies of these elements reduces the cellular immune function both in man and laboratory animals and increases the susceptibility to infection and other immunologically mediated diseases. Among the non-essential trace metals, lead, cadmium and mercury have been shown to be immunotoxic in experimental animals (Koller, 1980; Chandra and Dayton, 1982). However, in most of the studies on immunotoxicity, very high doses or the parenteral mode of administration

has been used which is of relatively little significance to possible human toxicity. Nevertheless, these studies show that toxic metals also affect immune responses that are of potential health significance.

One important aspect that has emerged from recent studies on trace elements is the existence of interactions among them. Trace elements interact between themselves and also with other dietary elements. The topic has been reviewed recently (The Task Group on Metal Interaction, 1978; Levander and Cheng, 1980; Abdulla, Nair and Chandra, 1985; Chowdhury and Chandra, 1987). However the effects and implications of this phenomenon on immune functions has not been studied. On the premise that essential trace elements have important immunoregulatory effects, and the known toxic elements are also toxic to the immune system, demonstration of such interaction among these two groups of elements would be of basic and applied significance.

Cadmium and zinc are two important members of the two trace element groups. They both belong to group IIB of the periodic table and thus have many physical and chemical similarities. They are usually found together in nature and also in biological tissues where they compete for binding sites in ligands like metalloenzymes and the heavy metal binding protein metallothionein. Thus they form an interesting toxic and essential trace element pair in the biological system. In the subsequent sections of this chapter, salient features of cadmium and zinc will be discussed with particular emphasis on the immune system effects. Metallothionein, which is involved in the metabolism of both cadmium and zinc, will be briefly

discussed, and the known interactions between cadmium and zinc will also be discussed.

## 1.2. CADMIUM - A TOXIC HEAVY METAL

Cadmium is an ubiquitous element to which everyone is constantly exposed at a low level. At birth cadmium is virtually absent from tissues, but it is gradually acquired from the environment, so that in a lifetime an average person living in an industrial society accumulates about 15 to 30 mg of cadmium in his body (Friberg, Piscator, Nordberg and Kjellstrom, 1974). Although the acute toxicity of cadmium on the lung and gastrointestinal tract has been known for over a century (Wheeler, 1876), it was only in 1950's that the danger of chronic exposure to cadmium was appreciated. In 1942 Nicaud, Lafitte and Gros from France reported an unusual number of cases of osteoporosis associated with an impairment of general health in an alkaline battery factory workers and suggested cadmium to be the causative agent (Friberg et al., 1974). A few years later in Sweden, Friberg (1948, 1950) conducted a survey on workers exposed to cadmium-oxide dust in an electrical battery plant and found a high number of cases of lung and kidney damage characterized by emphysema and low molecular weight proteinuria respectively. Since then, many similar investigations performed in several countries have confirmed the danger of chronic long-term cadmium exposure, and cadmium compounds became recognized as a serious occupational health hazard (Kostial, 1986).



The greatest concern over cadmium pollution was triggered by reports from Japan which showed that chronic cadmium poisoning was not restricted to industrial workers only, but can constitute a health hazard to the general population as well. A large population group in Japan was exposed to cadmium by contamination of food and water through a mine. The total daily intake of cadmium was about ten-fold greater than that in most parts of the world. In some areas the exposure was high enough to cause an epidemic of severe bone disease - the *Itai-itai disease*, (Friberg et al., 1974). Another case of cadmium exposure among the general population occurred in a village in England. The source of contamination was food grown in an old zinc-mining area. The level of exposure was comparable to that of Japan, liver cadmium levels were high in the exposed population and some evidence of kidney damage was noted, but none developed the typical features of *Itai-itai disease* (Carruthers and Smith, 1979; Inskip, Beral and McDowall, 1982).

Cadmium has no essential biological function. Although one preliminary communication has reported a growth supporting effect of cadmium in rats (Schwarz and Spallholz, 1976), there are no confirmatory reports substantiating this finding. So at present all evidence indicate that cadmium is only a toxic element and its presence in cells must be regarded as something to be minimized.

### 1.2.1. Source of cadmium exposure

Cadmium exposure can occur both in the industrial and general environment. Industrial processes that use cadmium include electroplating,

fabrication of alloys and solders, manufacture of paints where cadmium salts are used as pigments and plastics where it is used as a stabilizer, and in manufacture of cadmium-nickel batteries (Bernard and Lauwerys, 1986). Certain other industrial processes, like production of cadmium and its compounds, smelting and refining of zinc and lead ores, recovery of scrap metal, combustion of coal and oil, and disposal of sewage and sludge and of waste plastics produces cadmium as a byproduct (Webb, 1975). Workers in both types of industries are at potential danger of inhaling large amounts of cadmium, as the processes involved emit cadmium particles and increase its level in the air.

For the general population, cadmium exposure occurs mainly through food. However, cigarette smoke contains a significant amount of cadmium, and thus in heavy smokers this is an important additional source of cadmium. Drinking water and ambient air usually contain very little cadmium, and contribution to total body burden of cadmium from these sources are insignificant.

Volcanic activity and erosion of land has contaminated the earth's environment and polluted its food supply with cadmium since the beginning of life; but it is only in the last three decades that cadmium has been given a serious consideration as a food contaminant. As the industrial use of cadmium increased over the past thirty years, so did its level in the environment and consequently in the food chain (Fox, 1979; Ryan, Pahren and Lucas, 1982). Several studies have been conducted to estimate the average daily cadmium intake from food. The values are extremely variable depending on the geographical region, dietary habit and source of

contamination. Some foods like oysters, kidney and liver are known to contain amounts of cadmium considerably higher than most other foods. Diets rich in these foods may considerably increase the cadmium intake (Mahaffey, Corneliussen, Jelinek and Fiorino, 1975). The reported daily intake of cadmium from various countries shows values of 10 - 51  $\mu\text{g/day}$  for the United States, 10 - 20  $\mu\text{g/day}$  for the United Kingdom, 11 - 18  $\mu\text{g/day}$  for Sweden, 15  $\mu\text{g/day}$  for Belgium and 20 - 70  $\mu\text{g/day}$  for Japan (Buchet, Lauwerys, Vandevogde and Pycke 1983; Page, El-Amamy and Chang, 1986). For Canada, the average daily intake has been reported to be 67  $\mu\text{g}$  (Kirkpatrick and Coffin, 1974). It has been estimated that cadmium intake of 200-390  $\mu\text{g/day}$  for 50 years by a healthy 70 Kg man would result in kidney damage (Fox, 1983). Thus it can be derived that the current dietary cadmium content of normal people provides a safety margin of only 4 to 20 fold.

Various studies have documented the importance of cigarette smoking as a source of cadmium. Analysis of necropsy material from smokers show a higher body burden of cadmium in smokers as compared to non-smokers (Lewis, Jusko, Coughlin and Hartz, 1972; Hafin, Ewers, Jermann, Freier, Brockhaus and Schlipkoter, 1987). The amount of retained cadmium is also directly proportional to the number of cigarettes smoked. By *in vivo* measurement, the total body burden of cadmium was seen to be double in smokers as compared to non-smokers (Ellis, Vartsky, Zanzi, Cohn and Yasamura, 1979). In pregnant women who smoked, cadmium levels were elevated in their blood, as also in the placenta and fetal blood (Kuhnert, Kuhnert, Bottoms and Erhard, 1982). In a recent survey, blood cadmium level was seen to be increased in smokers with a dose-effect relationship

to the number of cigarettes smoked per day. Even ex-smokers had higher blood cadmium levels (Moreau, Lellouch, Juguet, Festy, Orssaud and Claude, 1983).

### 1.2.2. Metabolism of cadmium

The principal features of cadmium metabolism are its long biological half-life and interaction with other nutrients, particularly zinc. The long half-life is due to the lack of any homeostatic control mechanisms that can deal with the increasing intake of cadmium with age. The only protection the mammalian system offers against cadmium is through the synthesis of the intracellular metal-binding protein metallothionein.

The main routes of cadmium entry into the body are the gastrointestinal tract and the lung. Virtually the total amount of cadmium present in the body enters through these two organs.

Very little quantitative data are available on the absorption of cadmium in man. Most values are based on the analysis of human autopsy materials and estimates of dietary intake. These limited human data and supportive animal experiments indicate that the gastrointestinal absorption of cadmium is very low, and is generally between 3 and 8 percent of the intake (Perry, Thind and Perry, 1976). Cadmium is absorbed by a process of passive diffusion in the duodenum, jejunum and ileum (Sahagian, Harding-Barlow and Perry 1966; Sahagian, Harding-Barlow and Perry 1967). In the intestine, cadmium concentrates in the epithelial cells and much of it is subsequently lost when the cells are shed from the villi. However, the observation of enteropathy in *Itai-itai disease* patients (Murata, Hirono,

Sacki and Nakagawa, 1969) and in experimental animals fed large doses of cadmium (Richardson, Fox and Fry, 1974) suggest that when cadmium concentration in the villi crosses a critical level, the cells are damaged and a substantial amount of cadmium may enter the body.

Absorption of cadmium from the lung is much higher as compared to the gastrointestinal tract. The rate of absorption depends on the size and molecular form of the respirable particles of cadmium. The form present in cigarette smoke is usually more completely absorbed than those from industrial sources. One model based on human autopsy study estimates that 50 percent of inhaled cadmium from cigarette smoke is absorbed (Elinder, Kjellstrom, Friberg, Lind and Linnman, 1976). In non-smokers, inhalation contributes very little cadmium to the body. The absorbed fraction is also considerably less for other respirable sources. Generally about 25 percent of cadmium inhaled in ambient air is deposited in the lower respiratory tract, and about 13 to 19 percent of the inhaled cadmium is absorbed (Friberg et al., 1974).

After absorption cadmium is transported in blood. The mode of cadmium transport in blood is unknown, but it is thought that cadmium is transported in plasma bound to low molecular weight protein(s) and in erythrocytes bound to haemoglobin and other protein(s) (Nordberg, Piscator and Nordberg, 1971). Animal studies indicate that freshly absorbed cadmium is first taken up by the liver and incorporated into metallothionein. This cadmium-metallothionein complex is then slowly released into blood and taken up by other tissues, particularly kidneys (Kostial, 1986). In tissues, cadmium is again bound into metallothionein, which keeps the metal in a

non-ionic form that is non-toxic. Kidneys, for some unknown reason, produce the highest amount of metallothionein and concentrate the largest amount of cadmium. Liver also concentrates a considerable amount of cadmium, but the amount is generally much lower than kidneys. However, due to its larger weight the liver contains a significant fraction of the total body burden. In kidneys, a cadmium gradient occurs with the concentration in the outer cortex being twice that in the medulla (Livingston, 1972).

Cadmium, once stored in tissues, is released very slowly. Its biological half-life has been calculated to be 5-10 years in the liver and 16-33 years in the kidneys (Kjellstrom, 1971; Friberg et al., 1974). Very small amounts of cadmium are normally excreted from the body. The principal route of excretion is the urine and in normal man the urine cadmium concentration varies from  $< 0.5$  to  $2 \mu\text{g/liter}$ . The level is higher in smokers than in non-smokers. Also with increasing age, as tissue cadmium levels increase, the urinary output of cadmium also increases. Thus normally urinary cadmium levels reflect the total body burden of the metal (Kostial, 1986). However, when renal damage occurs due to very high kidney cadmium load, the excretion increases dramatically and may reach values over  $100 \mu\text{g/day}$  (Hallenbeck, 1986).

### 1.2.3. Toxic effects of cadmium

Cadmium is toxic to virtually every system of the body. Extensive information exists on the toxic effects of cadmium, however, for obvious reasons most of the data are from animal studies. Table 1.1 summarizes the

TABLE 1.1

## Toxic Effects of Cadmium

Type	Effects	Reference
Acute	Gastroenteritis	Nordberg et al., 1973
	Bronchitis and pulmonary edema	Friberg et al., 1974
	Reproductive system effect *	Parizek, 1956 & 1983
Chronic	Renal damage and proteinuria	Piscator, 1986
	<i>Itai-itai disease</i>	Friberg et al., 1974
	Chronic obstructive lung disease	Stanescu et al., 1977
	Essential hypertension *	Koop et al., 1982
	Carcinoma of lung and prostate *	Thun et al., 1985
	Teratogenesis *	Machemer et al., 1981
	Immunotoxicity *	Koller, 1980 Borgman et al., 1986
	Occasionally - Mild anemia,	Bernard et al., 1986
	Yellow coloration of teeth,	
	Nasal mucosal ulceration,	
	Anosmia, Liver damage	

\* Inconclusive evidence of occurrence in man

manifestations of acute and chronic cadmium toxicity. In this section the major features of cadmium toxicity are discussed, while in the next section (1.2.4) the present state of knowledge on the immune system effects of cadmium is reviewed.

Acute cadmium poisoning is rare in man. Massive oral and respiratory cadmium exposure has been reported following ingestion of foods contaminated by cadmium-plated utensils (Nordberg, Slorach and Stenstrom 1973) and from inhalation of cadmium containing fumes in industries (Bernard and Lauwerys, 1986). Symptoms of oral cadmium exposure were mostly referable to the upper gastrointestinal tract, and included nausea, vomiting, diarrhoea, abdominal cramps and rarely shock due to dehydration. Inhalation of cadmium fumes, which can occur in industries where cadmium is heated to a very high temperature resulting in the production of toxic cadmium-oxide fumes, causes bronchial and pulmonary irritation and sometimes fatal pulmonary edema (Stanescu, Veritet, Frans, Goncette, Roels, Lauwerys and Brasseur, 1977). Laboratory animals exposed to large doses of cadmium by oral or inhalation route also show similar manifestations, however, for some unknown reasons rodents tolerate large doses of oral cadmium without gastrointestinal reactions (Kostial, 1986).

Following parenteral administration of large doses of cadmium to animals, reproductive system effects are most prominent. In male rats, a single large dose of cadmium by injection characteristically causes haemorrhagic necrosis of the testis (Parizek and Zahor, 1956). The necrosis is caused by interference with testicular blood supply by cadmium (Gunn, Gould and Anderson, 1963a). Testicular necrosis can also be induced by



relatively low doses that do not damage other organs. In female rats, cadmium injection causes hyperemia of the ovary, atresia of the follicles and features similar to toxemia of pregnancy (Parizek, 1983). The cause of this predominant involvement of the reproductive organs is not known. In humans, no effects on reproductive organs have been reported, but it is hypothesized that cadmium may play a role in the development of pregnancy induced hypertension (Chisolm and Handorf, 1985).

Unlike acute cadmium toxicity, chronic cadmium toxicity is relatively more common. In cadmium exposure, all tissues acquire the metal, but kidneys concentrate the highest amount and renal damage is the major feature of cadmium poisoning. Even after respiratory exposure, kidneys are ultimately involved, although lung is the first organ affected. Proximal tubular cells are typically damaged in chronic cadmium toxicity resulting in a Fanconi type proteinuria characterized by loss of low-molecular weight proteins like  $\beta_2$ -microglobulin, retinol-binding protein and lysozyme; and amino acids, glucose and phosphate (Piscator, 1986). In cadmium exposed workers, increased urinary excretion of high molecular weight proteins like albumin, gamma-globulin, and transferrin are often detected as an isolated finding or in association with low molecular weight proteinuria (Bernard, Buchet, Roels, Masson and Lauwerys, 1979; Buchet, Roels, Bernard and Lauwerys, 1980). The loss of high molecular weight proteins probably results from an increased glomerular permeability together with decreased tubular reabsorption (Lauwerys, Bernard, Roels, Buchet and Viau, 1984). Renal dysfunction typically occurs when the average cortical concentration reaches 200 to 300  $\mu\text{g/g}$  wet weight, the amount varies depending on the type of cadmium compound and rapidity of administration (Ellis, Morgan,

Zanzi, Yasumura, Vartsky and Cohn, 1981; Roels, Lauwerys and Dardenne, 1983; Piscator, 1986). The precise mechanism of cadmium nephrotoxicity is not known. Normally, cadmium-metallothionein complex released from the liver into the blood is freely filtered through the glomeruli and subsequently taken up by the proximal tubular cells by pinocytosis. Inside the tubular cells cadmium is bound to newly formed metallothionein. It has been suggested that kidney damage is prevented as long as tubular cells produce enough metallothionein; beyond that the cells are damaged by non-metallothionein-bound cadmium ion, and low molecular weight proteins that are normally reabsorbed by proximal tubular cells begin to appear in urine (Friberg, 1984). It has also been proposed that the tubular epithelial cell membranes may be damaged during the process of cadmium-metallothionein complex absorption from the tubular lumen, and the cadmium-metallothionein complex itself may damage the tubular cells in addition to free ionic cadmium (Kostial, 1986; Suzuki and Cherian, 1987). The immune system has also been implicated in the pathogenesis of cadmium nephrotoxicity. In cadmium treated animals immune complex glomerulonephritis (Joshi, Dwivedi, Powell and Holscher, 1981) and circulating anti-glomerular basement membrane antibodies (Bernard, Lauwerys, Gengoux, Mahieu, Foidart, Druet and Weening, 1984) have been detected. The health implications of low-molecular proteinuria, which is otherwise asymptomatic, is a topic of debate. However, tubular dysfunction, once established, is irreversible and usually progressive; thus it is usually accepted as an early sign of cadmium toxicity (Lauwerys and Bernard, 1986). Recent studies in industrially exposed workers show that tubular dysfunction even appears at exposure levels that are currently accepted as

normal (Verschoor, Herber, Hemmen, Wibowo and Zielhuis, 1987; Jakubowski, Trojanowska, Kowalska, Gendek, Starzynski, Krajewska and Jajte, 1987).

Bone lesions characterized by osteomalacia, osteoporosis, and spontaneous fractures are usually late manifestations of severe cadmium poisoning. *Itai-itai disease* was an extreme manifestation of cadmium-induced osteomalacia. Typical features were bone pain, difficulty in walking, and spontaneous fracture. The disease typically affected middle-aged multiparous women (Friberg et al., 1974). Since concentration of cadmium in the bone does not increase even at a high body burden, and cadmium is not known to have any direct effect on bone, the effect of cadmium on bone in *Itai-itai disease* patients was probably secondary to renal tubular dysfunction with increased urinary loss of calcium and phosphate along with decreased dietary intake of protein and calcium and increased loss due to pregnancies.

Cadmium has also been linked to other diseases like essential hypertension and carcinoma of the lung and prostate. The hypothesis of a link between cadmium and hypertension was based on the observation that several anti-hypertensive drugs show increased binding to some transition elements like cadmium, copper and zinc. Of these, cadmium received most attention due to its affinity for the kidneys, the organ recognized for its role in controlling blood pressure (Perry, 1972). Rat studies clearly indicate that cadmium at a dose range of 0.1 to 20 ppm in drinking water can produce elevation of systolic and diastolic blood pressure, while doses above this range decreases the blood pressure (Perry, Erlanger and Perry,

1977; Perry, Erlanger and Perry, 1979; Koop, Glonek, Perry, Erlanger and Perry, 1982). In spite of clear evidence in animals, there is no direct proof of an association between cadmium and hypertension in humans. In an endemic area of high cadmium exposure in Japan; examination of a very large sample of the population did not reveal any relationship of blood pressure with the degree of pollution (Shigematsu, Minowa, Yoshida and Miyamoto, 1979).

The interest in cadmium as a possible carcinogen was generated by some early epidemiological studies showing an increased incidence of prostatic and lung cancer in occupationally exposed workers (Kipling, Waterhouse, 1967; Lemen, Lee, Wagoner and Blejer, 1976). But in most of these studies the number of observed cases were too small to permit a firm conclusion, and often the possible effects of cocarcinogens like tobacco and other industrial chemicals were not considered. However, in rats and mice, cadmium given by injection or at a large oral dose for a long period produces interstitial cell tumour in the testes or sarcoma at the site of injection (Haddow, Roe, Dukes and Mitchley, 1964; Bomhard, Vogel and Loser, 1987). Also prolonged inhalation of cadmium chloride fumes at exposure levels within the current occupational limits has been demonstrated to cause a dose dependent increase in lung cancer in rats (Takenaka, Oldiges, Konig, Hochrainer and Oberdorster, 1983). In contrast to definitive animal studies, results from human studies are inconclusive. A recent report of a study in which a large cohort of industrially exposed workers were examined, a definite increase in lung cancer was observed (Thun, Schnorr, Smith, Halperin and Lemen, 1985). However, in another similar study no association between cadmium and cancer was found

(Sorahan, 1987). Thus the association of cadmium and human malignancies is not yet established, but the evidence strongly suggests that cadmium is a carcinogen.

Other toxic effects often attributed to cadmium include teratogenicity and embryotoxicity (Machemer and Lorke, 1981; Machemer and Lorke, 1981). Cadmium has also been reported to cause mild anemia, yellow coloration of teeth, anosmia, ulceration of nasal mucosa and signs of liver damage (Bernard and Lauwerys, 1986).

#### 1.2.4. Effect of cadmium on immune responses

The effects of cadmium compounds on the immune system have been studied extensively in different animal species in recent years and the topic has been reviewed (Koller, 1980; Exon and Koller, 1986). Cadmium has been shown to significantly affect the immune responses, however a review of the literature reveals many inconsistencies. The antibody response and cell-mediated immunity have been shown to be both enhanced and suppressed following cadmium treatment. The effect varies depending on the dose, duration and route of cadmium treatment, temporal relationship between cadmium treatment and administration of antigen and the species of animal tested. This is not unexpected as cadmium absorption from different sites is known to vary and the effect of a toxic element on an intricately controlled system like the immune system would be expected to be dose dependent.

Although the results of different studies are ambiguous, cadmium is generally immunosuppressive (Koller, 1980). Primary and secondary immune

responses against heterologous antigens are suppressed in rats and mice treated with large oral doses of 300 ppm cadmium in drinking water for 10 weeks (Koller, Exon and Roan, 1975) or by cadmium injection (Bozelka, Burkholder and Chang, 1978; Shippee, Burgess, Ciavarra, DiCapua and Stake, 1983) or inhalation (Graham, Miller, Daniels, Payne and Gardner, 1978; Krzystyniak, Fournier, Trottier, Nadeau and Chevalier, 1987). In contrast, low oral doses of 25 or 50 ppm cadmium in drinking water has been reported to have no effect on primary antibody forming cells (Muller, Gillert, Krause, Jautzke, Gross and Diamantstein, 1979; Wesenberg and Wesenberg, 1983), or to increase (Malave and DeRuffino, 1984) or to decrease (Blakley, 1985; Borgman, Au and Chandra, 1986) their number. Also parenteral administration of 0.15 mg of cadmium in mice before antigen challenge increases IgM and IgG antibody production (Koller, Exon and Roan, 1976), but at doses of 1.8 mg/kg body weight suppresses antibody response due to direct inactivation of B cells (Fujimaki, 1985). Cadmium injection initiated 7 days before antigen administration suppresses antibody synthesis, but it enhances the response when initiated 14 days earlier (Jones, Williams and Jones, 1971).

Other data suggest that cellular immune functions are also affected by cadmium. Delayed cutaneous hypersensitivity reaction which is a classical *in vivo* test for cell-mediated immunity is impaired by cadmium in mice (Muller et al., 1979). However, the *in vitro* tests of cell-mediated immunity do not show uniform effects following cadmium exposure. Lymphocyte transformation *in vitro* to mitogens like phytohaemagglutinin (PHA), concanavalin A (Con A) and pokeweed mitogen (PWM) is a commonly employed assay of cell-mediated immunity. The plant lectins PHA and Con

A activate T cells, and PWM activates both T and B cells. In some studies, lymphocytes from mice and rats exposed to cadmium have been shown to have increased response to T cell mitogens (Muller et al., 1979; Malave and DeRuffino 1984), whereas in others no change (Wesenberg and Wesenberg, 1983; Blakley, 1985) or a decrease (Gaworski and Sharma, 1978) in response has been observed. B cell responses are generally increased in cadmium treated animals (Muller et al., 1979; Koller, Roan and Kerkvliet, 1979; Blakley, 1985). These conflicting reports indicate that cadmium can impair some parameters of the immune response while augmenting others.

Other immune cells like the macrophages and polymorphonuclear leukocytes are also adversely affected by cadmium. Phagocytic capacity of these cells is reduced (Loose, Silkworth and Warrington, 1978). Macrophages from cadmium treated animals produce less macrophage migration inhibitor factor, a lymphokine (Kiremidjian-Schumacher, Stotzky, Likhite, Schwartz and Dickstein, 1981), are sluggish in movement and less responsive to macrophage migration inhibitor factor (Kiremidjian-Schumacher, Stotzky, Dickstein and Schwartz, 1981). Also, cadmium treatment inhibits the ability of immune macrophages to destroy cancer cells *in vitro* (Nelson, Kiremidjian-Schumacher and Stotzky, 1982). In addition, large doses of cadmium affects the maturation of lymphoid cells and results in an increase in large immature cell types (Ohsawa and Kawai 1981; Burchiel, Hadley, Cameron, Fincher, Lim, Elias and Stewart, 1987). As a result of generalized immunosuppression, cadmium treated animals show increased susceptibility to bacterial, viral and protozoal infection (Cook, Hoffmann and DiLuzio, 1975; Gainer, 1977; Exon, Patton and Koller, 1979; Exon, Koller and Kerkvliet, 1979). Incidence of cancer is also increased in

a dose dependent fashion (Takenaka et al., 1983). Development of cancer is probably multifactorial, but cadmium induced alteration of immune function may contribute to its pathogenesis.

The implications of cadmium immunotoxicity on human health are presently unknown. However, animal observations suggest that cadmium may well be immunotoxic in man. *In vitro* studies with human blood exposed to cadmium chloride show that lymphocytes accumulate cadmium far in excess of other cells. When blood is exposed in culture to cadmium at levels comparable to blood cadmium levels found in cases of occupational human exposure, the lymphocytes accumulate approximately 800-fold more cadmium than red cells (Hildebrand and Cram, 1979). The cause of such selective uptake of the metal is not known, however, this observation provides one possible explanation for the finding of altered immune response in experimental animals exposed to cadmium, as lymphocytes are the key cellular components of the immune function. A similar situation may also exist in the human population exposed to high levels of cadmium in industries or through heavy cigarette smoking.

### 1.3. ZINC - AN ESSENTIAL TRACE ELEMENT

Zinc has long been known to be an essential trace element for the growth and development of living organisms. The first evidence of its essentiality in biology was the demonstration over a century ago that zinc is required by the fungus *Aspergillus niger*. After a lapse of almost sixty years, the essentiality of zinc for the higher forms of plant life was established. Subsequently many investigations revealed the presence of zinc



in plants and animals in concentrations often comparable with those of iron and usually much greater than those of other trace elements (O'Dell, 1984). However, the essentiality of zinc for mammals was not established until 1934, because of early difficulties in producing a zinc deficient diet for laboratory animals and the apparent absence of natural zinc deficiency states. Todd, Elvehjem and Hart (1934) first produced unequivocal evidence that zinc is an essential dietary element for the rat. Subsequently other workers established the essentiality of zinc in other animal species including, rodents, poultry and ruminants (Luecke, 1984).

Interest in zinc in human nutrition followed the work of Prasad and coworkers (Prasad, Miale, Farid, Sandstead and Schulert, 1963; Prasad, Miale, Farid, Sandstead, Schulert and Darby, 1963) who in early 1960s provided the first evidence for the occurrence of nutritional zinc deficiency in the human. Subsequently acrodermatitis enteropathica was described as a genetic defect of zinc absorption; and it was appreciated that nutritional and conditional zinc deficiency may complicate many disease states in man including steatorrhea and other malabsorption syndromes, patients on total parenteral nutrition, penicillamine therapy, alcoholism, burns, sickle cell anemia, Down's syndrome and chronic renal disease (Prasad, 1984; Hambidge, Casey and Krebs, 1986). The importance of zinc in human nutrition was further highlighted by the observation that even in the free-living population in an industrialized country, zinc nutriture was marginal or deficient in some groups, like the elderly, some infants, pregnant teenagers, institutionalized individuals and the poor (Sandstead, 1973).

In humans zinc is essential for almost every organ system of the body. The clinical manifestations of zinc deficiency include bullous-pustular dermatitis, alopecia, diarrhoea, mental disturbances, male hypogonadism, poor appetite, decreased taste acuity, abnormal dark adaptation, delayed wound healing and intercurrent infections as a result of disordered cell-mediated immunity (Hambidge, Casey and Krebs, 1986). Animal studies also substantiate these findings. The role of zinc on immune functions has been studied extensively in recent years and reviewed (Chandra, 1983; Chandra and Puri 1985; Fraker, Gershwin, Good and Prasad, 1986; Fraker, Jardieu and Cook, 1987; Chandra, 1987). In a subsequent section (1.3.2), the role of zinc in immunity is discussed in greater detail. To place the information into perspective, the general biological and cellular functions of zinc are briefly discussed in the next section.

### 1.3.1. Biological functions of zinc

Zinc has the ability to form stable complexes with the side chain of proteins, a property that is relevant to its biological functions. The involvement of zinc in enzyme functions and structures is the best known function of this metal. Vallee (1983) conjectured that the biological abundance of zinc, its ability to interact with widely varied coordination geometries and its resistance to oxidation-reduction have generated selective evolutionary pressure for it to serve as a biological catalyst. Carbonic anhydrase was the first zinc metalloenzyme to be identified and purified (Keilin and Mann, 1940). Subsequently many other zinc enzymes have been identified, and if related enzymes from different species are included, then over 200 zinc metalloenzymes are now on record (Vallee,

1983; Prasad, 1984). Zinc is postulated to have a catalytic, structural and regulatory role in these proteins. Among others, zinc is also needed for enzymes involved in nucleic acid metabolism like DNA polymerase, RNA polymerase, reverse transcriptase and nucleoside phosphorylase (Lieberman, Abrams, Hunt and Ove, 1963; Ballester and Prasad, 1983). Involvement of these enzymes in zinc deficiency would thus be expected to have a profound effect on rapidly proliferating cells like the cells that take part in immune responses.

Zinc also has an important structural role at other sites. It is speculated that zinc may form mercaptides with the thiol group of proteins, link the phosphate moiety of phospholipids or interact with carboxyl groups of sialic acid or proteins on plasma membranes resulting in a change in fluidity and stabilization of plasma membranes (Chvapil, 1976). Similar effects can also occur at other biomembranes. Substantial quantities of zinc are present in RNA and DNA where it is thought to stabilize the structure of the molecules (Hambidge, Casey and Krebs, 1986). Some regulatory proteins involved in gene expression also contain the zinc atom which stabilizes the three dimensional structure of the DNA binding domain of these proteins (Kadonaga, Carner, Masiarz and Tjian, 1987; Klug and Rhodes, 1987). Thus in zinc deficiency, membrane functions and nucleic acid metabolism are altered, which partly explain the immunological alterations seen in zinc deficiency.

### 1.3.2. Zinc and Immunity

The effects of zinc deficiency have been studied extensively in patients with acrodermatitis enteropathica (Chandra, 1980) and other states

of acquired zinc deficiency (Golden, Jackson and Golden, 1977; Pekarek, Sandstead, Jacob and Barcome, 1979; Chandra and Puri 1985; Tapazoglou, Prasad, Hill, Brewer and Kaplan, 1985), and rodents fed zinc deficient diet (Fernandes, Nair, Onoe, Tanaka, Floyd and Good, 1979; Fraker, Zwickl and Leucke, 1982). Zinc deficiency affects various immune responses, but cell-mediated immunity is affected more than others. Both in man and laboratory animals, the lymphoid tissues particularly the thymus, are atrophied. The effect is more prominent in the cortical area of the thymus and thymus-dependent areas of peripheral lymphoid tissues. In the peripheral blood, absolute number of lymphocytes, T cells and CD4<sup>+</sup> helper/inducer T cells are reduced in number. Furthermore, lymphocytes from zinc deficient subjects or animals show reduced blastogenic response to T cell mitogens PHA and Con A. As a result of altered cellular immune profile and T cell function, patients with zinc deficiency are usually anergic to common recall antigens. Sensitivity to chemical sensitizing agents is also suboptimal. Other cellular functions like the natural-killer cell activity and antibody-dependent cell-mediated cytotoxicity are also reduced in zinc deficiency, but these findings are not consistent. The effects of zinc deficiency on cellular immune functions usually develop rapidly following depletion, and replenishment with zinc reverses the effects.

Studies on the impact of zinc deficiency on antibody production and other aspects of humoral immunity show that B cell function is also affected by zinc. In zinc deficient animals, both primary and secondary antibody responses to heterologous antigens are reduced; and proliferative response of lymphocytes to the B cell specific mitogen, lipopolysaccharide

is reduced. Zinc deprivation during in utero development in mice severely affects the immune cells and cause a persistent state of immunodeficiency in the offspring that can even be transferred to subsequent generations, suggesting that zinc plays an important role in immunologic ontogeny (Beach, Gershwin and Hurley, 1982).

Zinc also affects the function of polymorphonuclear leukocytes (Weston, Huff, Humbert, Hambidge, Neldner and Walravens, 1977). In zinc deficiency, chemotactic response of these cells is reduced which is corrected after replenishment. However, following excessive zinc intake in human subjects, polymorphonuclear function has been reported to be reduced along with reduced blastogenic response of T cells to mitogens. Altered lipid profile in blood and leukocyte membranes has been suggested as an explanation for the observation of suppressed immune response in zinc excess (Chandra, 1984a).

As a result of altered immune function, zinc deficient laboratory animals usually show increased susceptibility to common infectious agents. Zinc deficiency induced immunosuppression also delays the onset and slows the progression of spontaneous autoimmune disease in NZB mice resulting in enhanced rate of survival and increased life span (Beach, Gershwin and Hurley, 1981). Depressed immunity may also be largely responsible for the increased susceptibility to infections and poor wound healing seen so often in congenital and acquired zinc deficiency states in human.

Various hypotheses have been put forward to explain the effect of zinc on immunity and some of these have been proven experimentally. Serum thymic hormone activity is usually reduced in zinc deficiency and is

promptly corrected by dietary zinc supplements. Experiments suggest that zinc is an essential cofactor for thymulin, one of the thymic hormones (Dardenne, Pleau, Nabarra, Lefrancier, Derrien, Choay and Bach, 1982; Dardenne, Savino, Wade, Kaiserlian, Lemmonier and Bach, 1984). Reduced thymic hormone activity explains in part the immunological effects of zinc deficiency. Experimental evidence suggests that even in acquired immunodeficiency syndrome, some of the immunological defects may be partly due to decreased zinc saturation and consequent reduction in thymulin level (Farbis, Mocchegiani, Galli, Irato, Lazzarin and Moroni, 1988). Zinc deficiency is also associated with an elevated level of free cortisol concentration in the blood, which may have a lympholytic effect (DePasquale-Jarideu and Fraker, 1980). Zinc being an essential cofactor of a variety of metalloenzymes is essential for cellular multiplication. Thus in deficiency, cellular multiplication is decreased and the numbers of T and B cells produced during the normal resting phase as well as during antigen stimulation are reduced.\*

Due to its varied immunological effects zinc has been tried in various immunodeficiency conditions. Elemental zinc supplementation at a dose of 50-100 mg daily for 4-6 weeks has been shown to have a beneficial effect on depressed immune responses that are common in the aged (Duchateau, Delepesse, Vrijens and Collet, 1981; Chandra 1984b). At a dose of 150 mg/day for one month zinc even stimulates lymphocyte response of normal healthy adults (Duchateau, Delepesse and Vereecke, 1981). From these studies it appears that zinc may also exert an immunoregulatory pharmacological effect.

#### 1.4. METALLOTHIONEIN

Metallothionein was first discovered as a cadmium and zinc binding protein in the kidneys of horses (Kagi and Vallee, 1960). Subsequent studies have characterized the structure, function and distribution of the protein to a great extent, and the topic has been reviewed recently (Hamer, 1986; Dunn, Blalock and Cousins, 1987):

Metallothioneins are low-molecular weight intracellular proteins that selectively bind heavy metal ions such as the nutritionally essential trace elements zinc and copper and the potentially toxic elements cadmium and mercury. Metal free protein is called "thionein" and that bound to one or more metals is called metallothionein. Sometimes more specific terms like (cadmium)-metallothionein, (zinc)-metallothionein, or (cadmium,zinc)-metallothionein are used when the metal content is known. Vertebrate tissues contain two forms of metallothionein, metallothionein-I and metallothionein-II. Metallothionein-I has further subforms like metallothionein-IA, metallothionein-IB etc. Metallothionein-II is usually the predominant form of the protein.

##### 1.4.1. Physiology and biochemistry of metallothionein

Metallothioneins are present in a broad range of eukaryotic species in many different tissues and cell types, with highest concentrations in the liver, kidneys, and intestine. The protein has also been reported to occur in invertebrates and microorganisms. Within cells metallothioneins are located mainly in the cytoplasm but the protein has been found within hepatic and renal nuclei as well. Synthesis of metallothionein is induced by

various agents like metal ions, hormones, and other factors. The synthesized metallothionein binds to heavy metals and degrades with a half-life of 12 to 84 hours. The rate of degradation varies, depending on the metal content. Thionein is most rapidly degraded, followed by zinc-metallothionein, cadmium-metallothionein and copper-metallothionein in that order. After degradation, the bound metal is released and the amino acids enter the cellular amino acid pool. It is thought that the degradation process occurs mainly in the lysosome. Metallothionein can also release the metal without being degraded and can readily exchange metals with either free metal ions in the cytoplasm or ions bound to other ligands.

Metallothionein is a low molecular weight (less than 10,000 D) protein with a high content of cysteine; and no aromatic amino acids, histidine, or disulfide bonds. Each molecule contains 4-12 metal atoms. A typical thionein is a 61 amino-acid peptide containing 20 cysteines, 7-10 serines, and 6-8 lysines. The majority of cysteine residues are present in Cys-X-Cys and Cys-Cys sequences, where X is an amino acid other than cysteine. Isoforms of metallothioneins generally have amino acid replacements outside these sequences. Metals are associated with thionein exclusively through thiolate bonds to all 20 cysteine residues. The tertiary structure of metallothioneins is characterized by two domains, an  $\alpha$ -domain within the carboxyl-terminal end extending from amino acid 31 to 61, and an amino-terminal  $\beta$ -domain extending from residue 1 to 30. Both domains are globular to give the whole molecule an ellipsoid shape. In both domains, the polypeptide chain makes three reverse turns to spiral around metal ions (Furey, Robbins, Clancy, Winge, Wang and Stout, 1986). The  $\alpha$ -domain contains 11 cysteine residues and binds 4 atoms of zinc or cadmium or 6



atoms of copper. The  $\beta$ -domain contains 9 cysteine and binds 3 atoms of zinc or cadmium or 6 atoms of copper. Zinc and cadmium preferentially fill the  $\alpha$ -domain first and then the  $\beta$ -domain; whereas copper saturates the protein in reverse order. The affinities of metals for binding to metallothioneins differ; that of copper is 100-fold greater than cadmium, which in turn is about 1000-fold greater than zinc. The metal composition of native metallothioneins are heterogeneous, even metal induced metallothioneins do not contain exclusively the metal used as an inducer; but it is not known whether more than one metal type can bind to one domain.

#### 1.4.2. Role of metallothionein in metal metabolism

The function of metallothionein has been debated ever since its discovery. Metallothioneins appear to function as detoxifiers, regulators and to some extent as carriers of heavy metals. *In vitro* studies show that cell lines that do not synthesize metallothioneins are more susceptible to toxic effects of heavy metals, whereas cells that show resistance produce higher than normal levels of metallothioneins (Crawford, Enger, Griffith, Griffith, Hanners, Longmire, Munk, Stallings, Tesmer, Walters and Hildebrand, 1985). In intact animals pretreatment with cadmium which is known to induce metallothionein synthesis also reduces the toxic effects of cadmium (Min, Hatta, Onosaka, Ohta, Okada and Tanaka, 1987). Metallothionein seems to be well suited for detoxifying cadmium. It is induced readily by cadmium, binds cadmium with high affinity, becomes more stable after binding, and degradative release of the metal results in its reassociation with other metallothioneins. This cycle apparently keeps

cadmium in a relatively non toxic form within cells. Other potential detoxification functions not directly related to metal include free radical scavenging and protection against ultra-violet and X-ray damage. Metallothioneins can also act as a storage site for nutritionally essential metals like copper and zinc and regulate their metabolism by modulating their movement within cells either directly through donation of these ions to metal requiring apoenzymes or metabolic compartments like biomembranes, or indirectly by regulating their free or available concentrations. The level of metallothionein varies in response to dietary and physiological changes. The inverse relationship between intestinal metallothionein and absorption of copper and zinc suggests that the protein may also regulate intestinal absorption of these metals. A carrier function of metallothionein is not clearly demonstrated, but the presence of low concentration of the protein in plasma, urine, and bile suggests that the protein may also transport metals in the body (Danielson, Ohi and Huang, 1982).

The best known regulators of metallothionein synthesis are heavy metals like cadmium, copper, zinc and mercury. They induce metallothionein synthesis primarily by increasing the rate of metallothionein gene transcription. Since the metal ion itself initiates the transcriptional event, the synthesis of metallothionein is regulated by a feedback mechanism. Metallothionein synthesis is also induced by hormones including glucocorticoids, glucagon, and epinephrine and by a number of other factors like cyclic-AMP, interferon, and interleukin-1. They also probably act at the metallothionein gene level. Any acute stress also

induces metallothionein synthesis perhaps through the above humoral agents (Dunn, Blalock and Cousins, 1987).

### 1.5. INTERACTIONS BETWEEN CADMIUM AND ZINC

Cadmium and zinc have many physical and chemical similarities as they both belong to group IIB of the Periodic Table. They are usually found together in the ores and also in mammalian tissues, and compete with each other for various ligands. Thus interaction between these two elements in the biological system is likely. Hill and Matrone (1970) hypothesized that elements whose physical and chemical properties are similar will act antagonistically to each other biologically. Elements of similar properties probably compete for the same transport and storage sites in the cells, and displace each other from reactive enzymatic and receptor proteins.

Parizek (1957) first demonstrated that a large dose of zinc could prevent cadmium induced testicular necrosis in rats when given together with cadmium. Webb (1972) showed that pretreatment with zinc was also protective against testicular effects of cadmium. Subsequent studies have confirmed these findings and extended the interaction to other toxic effects of cadmium like mortality (Shippee et al., 1983), growth suppression (Powell, Miller, Morton and Clifton, 1964; Ahokas, Dilts and LaHaye, 1980), nephrotoxicity (Watanabe, Nonomura, Tanahashi, Sugitani and Yamada, 1985), carcinogenicity (Gunn, Gould and Anderson, 1963b), hypertension (Perry, Erlanger and Perry, 1977), teratogenesis (Warner, Sadler, Tulis and Smith, 1984; Marlow and Freeman, 1987) and inhibition of DNA replication and repair (Nocentini, 1987). The biochemical mechanism(s) of cadmium-zinc

interaction is unknown, but various cellular and subcellular processes like the ratio of cadmium to zinc, induction and synthesis of metallothionein, binding characteristics of metallothionein, alteration of absorption, kinetic parameters, and tissue displacement of one metal by another and competition at the level of zinc containing metalloenzymes may be involved in the interactions.

In animals, cadmium treatment causes an increase in hepatic and renal zinc concentration at the expense of other organs (Petering, Johnson and Stemmer, 1971; Roberts, Miller, Stake, Gentry, and Neathery, 1973). Marcus (1982) demonstrated that zinc treatment affects the kinetic parameters of cadmium and alters movement of cadmium from one body compartment to another. Alterations of cadmium and zinc distributions in the body, which are probably mediated by metallothioneins, may be a mechanism for the interaction between cadmium and zinc.

In *in-vitro* studies, cadmium has been shown to reduce the activity of several zinc dependent enzymes (Vallee and Ulmer, 1972). It is possible that the toxicity of cadmium is, at least partly, due to a competition between cadmium and zinc at cofactor sites in enzymes requiring zinc. Tubular proteinuria that occurs in chronic cadmium poisoning may be explained by decreased activity of certain zinc requiring enzymes like alkaline phosphatase and leucine aminopeptidase, which are located in the brush border of proximal tubular epithelial cells and speculated to be involved in tubular reabsorption of proteins (Wachsmuth and Torhorst, 1974). In cadmium treated rats and pigs reduced activity of these enzymes has been observed (Elinder and Piscator, 1978).

Studies in rats show that a low level of cadmium produces severe manifestations of zinc deficiency when the diet is deficient in zinc, but not when the diet is adequate (Petering et al., 1971). The ratio of cadmium and zinc in the diet and also in the tissues is probably also a determining factor of cadmium toxicity and the interaction.

The implications of these interactions between cadmium and zinc are unknown, but their occurrence under controlled laboratory conditions suggests that similar events may also occur in free-living humans and animals, and thus is of potential health significance.

## CHAPTER 2

### RATIONALE AND OBJECTIVES

#### 2.1. RATIONALE

Cadmium is an ubiquitous toxic metal to which everyone is exposed at low levels. It is toxic to every organ system of the body. Nephrotoxicity is the characteristic feature of cadmium toxicity and has been studied extensively both in man and laboratory animals. Animal studies show that cadmium is also toxic to the immune system. However, in most of the studies on cadmium immunotoxicity, parenteral modes of cadmium or large oral doses had been used which bear little resemblance to possible human cadmium exposure. Also, the results were inconsistent and varied depending on the dose, duration and route of cadmium treatment. The principal objective of the present study was to characterize the effects of a relatively low dose of oral cadmium treatment on the immune system of mice. Mice were chosen as the experimental model as their immune system is well characterized and developmentally, structurally and functionally the murine immune system is remarkably similar to that of man. Since cadmium is known to have a long biological half-life, the study period was extended after cessation of cadmium treatment to look for any persistent or delayed effect of cadmium toxicity.

Zinc and cadmium have many physical and chemical similarities and they are usually found together in nature and also in mammalian tissues where they compete for binding sites in ligands like enzyme and other structural proteins. Both also bind to the heavy metal binding protein metallothionein. Studies have previously shown that many manifestations of acute and chronic cadmium toxicity in animals can be prevented by increasing the zinc status of the host. Thus it is possible that the immunological effects of cadmium may also be related to zinc status, and administration of a pharmacologic dose of zinc may prevent or correct the effects. The other principal objective of the study was to characterize the effect of a moderately large dose of zinc on cadmium-induced immunopathology. Demonstration of such an interaction at the cellular level would be of basic and applied significance.

## **2.2. OBJECTIVES**

The objectives of the present study are summarized as follows -

### **2.2.1. Primary objectives**

1. To study the effects of a relatively low dose of oral cadmium treatment on the immune system of mice.
2. To study the effect of a moderately large dose of zinc on cadmium-induced immunopathology.

### **2.2.2. Secondary objectives**

1. To look for an immunological mechanism of renal toxicity of cadmium.

2. To study the effect of cadmium on renal morphology and ultrastructure.
3. To study the tissue cadmium level and distribution after treatment.
4. To study the interaction between cadmium and zinc on renal morphology and tissue cadmium level.



## CHAPTER 3

### DESIGN OF THE STUDY

A series of four experiments were performed to study the immunological effects of a relatively low dose of cadmium, and the interaction between cadmium and zinc on the immune system of mice. In the four experiments, the basic study design was identical, however, the parameters examined varied. In essence, one experiment led to questions which were addressed in the next experiment. In the following sections the design of the study and the four experiments are outlined. Experimental procedures are described in chapter 4.

#### 3.1. EXPERIMENTAL GROUPS AND TREATMENT SCHEDULES

Male six week old C57BL/6 mice were used in the study. They were divided into six experimental groups, some of which received 50 ppm cadmium and/or 500 ppm zinc in drinking water. Other variables such as diet and housing, were identical for all groups. The design of the experiment is shown in Fig. 3.1. Group I received no treatment; group II received 50 ppm cadmium (cadmium chloride, No. B 10064-34, BDH Chemicals, Toronto) in deionized distilled water for 3 weeks and no treatment for the next 3 or 6 weeks; group III received 50 ppm cadmium together with 500 ppm zinc (zinc chloride, No. Z-0150, Sigma Chemical Company, St. Louis, Missouri) for 3 weeks and 500 ppm zinc for next 3 or

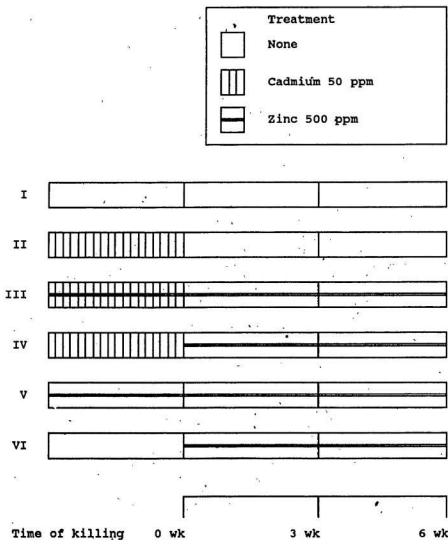


FIG. 3.1  
Design of the Experiment.

6 weeks; group IV received 50 ppm cadmium for 3 weeks and 500 ppm zinc for the next 3 or 6 weeks; groups V and VI received zinc in amounts equivalent to that given to groups III and IV but no additional cadmium. The treated waters were analyzed for cadmium and zinc content to ensure that the desired amount of cadmium and zinc was present. The water consumption of the animals was monitored and was comparable for all the groups.

Animals were killed for examination immediately after 3 weeks of cadmium treatment and at 3 and 6 weeks after cessation of treatment. The examination periods are termed 0, 3 and 6 week.

In each examination period there were 8 to 10 mice from each of the six groups. However, for 0 week there were four experimental groups as groups II and IV, and groups I and VI were identical at this point.

This design allowed study of the effects of a relatively low dose of cadmium immediately after cessation of treatment and at two time periods subsequently (group II), and also the effects of a large dose of zinc given together with and following or only following cadmium treatment (group III and IV). The two groups (group V and VI), which received equivalent amount of zinc as groups III and IV but no additional cadmium were included which allowed study of zinc *per se* on the immune system. The no treatment group (group I) gave the baseline values in this experimental setting.

Low dose of cadmium and the oral route of exposure were chosen as they are more relevant from the standpoint of possible human cadmium

toxicity. Since cadmium is known to have a long biological half life, the study period was extended after cessation of cadmium treatment. Cadmium was used at a dose of 50 ppm as previous studies have shown that at this dose the immune system of mice is affected, but the animals do not show any clinical manifestations of toxicity (Borgman, Au and Chandra, 1986). A large dose of zinc was used as zinc is known to be relatively non-toxic and it was postulated that a large amount of zinc would be required to counteract cadmium immunotoxicity. Both cadmium and zinc were given in drinking water as it is an easy and controllable mode of treatment in experimental animals.

### 3.2. SERIES OF EXPERIMENTS

A series of four experiments were performed. In the first experiment, animals from all groups and observation periods were studied, but in subsequent experiments, groups and observation periods were reduced with focus laid on the groups and time periods in which abnormalities were expected. The expectation was based on the findings from the previous experiments.

#### 3.2.1. Experiment 1

In this experiment mice from all groups and examination periods were studied. Weight of the mice and their food consumption was monitored on a weekly basis throughout the experiment. The liver, kidneys, spleen and thymus were weighed. Lymphocytes were counted in the blood, spleen and thymus. Direct plaque-forming cell response, which assays IgM antibody

production against heterologous antigens, was measured in the spleen. Proliferative response of spleen cells to T cell mitogens was also assayed at 0 and 3 week. The kidneys were examined for IgG and complement C3 deposition by immunofluorescence staining. At 0 and 6 week, transmission electron microscopic examination of kidney sections was performed and the liver and kidneys were examined for cadmium content.

### 3.2.2. Experiment 2

In this experiment the observation period of 6 week and treatment groups IV and VI were omitted, as no delayed or persisting effect of cadmium on the immune system was observed in experiment 1. Weight of mice and food consumption were recorded, and the spleen was weighed and lymphocytes counted. Indirect plaque-forming cell response, which assays IgG antibody production on antigen challenge, was measured in spleen cells.

### 3.2.3. Experiment 3

In experiment 3, the groups studied were similar to experiment 2, but the observation period was limited to 0 week, as in the previous experiments immunological changes were seen only to occur immediately after cessation of cadmium treatment. Weight of mice and food consumption was recorded. The spleen was weighed and lymphocytes counted. The number of helper and suppressor T cells in the spleen was counted by immunofluorescence technique using appropriate monoclonal

antibodies. Concanavalin A induced suppressor cell activity in spleen was also assayed.

#### 3.2.4. Experiment 4

In this experiment, the groups studied were similar to experiment 3. As in previous experiments, weight of animals and food consumption was measured, spleens weighed and the lymphocytes counted. The number of B cells in the spleen was counted by immunofluorescence technique using polyclonal purified antibody. Lymphocytes from the spleen were isolated and natural-killer cell activity measured by chromium release assay, and antibody production on pokeweed mitogen stimulation measured by enzyme-linked immunosorbent assay. The liver was analyzed for trace element levels.

#### 3.3. TIME FRAME

The four experiments were performed sequentially one after another. Since a large number of animals, ranging from 40 to 160, were involved in each series, each experiment was spread out over 2 to 4 months. On two or three days a week, 4 to 6 animals were started on the experimental protocol. Also on the day of killing, the same number of animals were handled simultaneously. At least one animal from each group was included on each day.

## CHAPTER 4

### METHODS

#### 4.1. HOUSING AND FEEDING OF MICE

Male C57BL/6 mice (Charles River Canada, Montreal, Quebec) were used in the experiments. They were maintained in the animal care facility of the Faculty of Medicine, Memorial University of Newfoundland. The mice were housed singly in wire meshed hanging cages or in plastic cages with bedding. In experiment 1, 2 and 3 the former type of housing was used, and in experiment 4 the latter type was used. The change was forced due to availability of cages, however, this would be of no significance as in each experiment only one type of housing was used. The environment of the animal rooms was controlled at 22-24°C temperature, 45-55% relative humidity, and 0800 to 2000 lighting hours. Standard guidelines for the care and use of experimental animals were followed throughout (Canadian Council on Animal Care, 1984), and the protocol was approved by the University Animal Care Committee.

Mice were purchased at ages of either 4 or 5 weeks. They were acclimatized for 1 or 2 weeks in the animal care facility during which they had free access to ordinary drinking water and food, and then put in one of the experimental groups at random. All mice were 6 weeks old at the beginning of the experiment. The mice were fed a non-purified diet

(Rodent Chow No. 5012, Ralston Purina, St. Louis, Missouri). The food pellets were analyzed for trace element contents and found to contain 0.09  $\mu\text{g}$  cadmium/g and 43.3  $\mu\text{g}$  zinc/g. Other constituents as indicated in the label are shown in Table 4.1. The mice also had free access to drinking water; water provided to some animals contained additional cadmium or zinc. The treatment schedules are shown in Fig.3.1 (page 40). Throughout the experiment, the weight and general health of animals and food disappearance was closely monitored and recorded. In experiment 1 the measurement was done at weekly intervals, but in the subsequent experiments, food disappearance was measured for a seven day period immediately preceding the killing.

#### 4.2. KILLING OF MICE AND COLLECTION OF TISSUES

Mice were anesthetized with ether, killed by cervical dislocation and the abdominal skin was wetted and the cavity opened. Anatomical attachments of the spleen, thymus, liver and kidneys were severed and the organs removed and transferred to preweighed containers which were weighed again to obtain the organ weights. The thymus was suspended in normal saline, fatty tissue removed and the organ dried with tissue paper before weighing. A single electronic balance (Mettler PC 180, Greinfensee, Zurich, Switzerland) was used throughout the studies.

In experiments where spleen cells were used for culture work, spleen was collected with full aseptic precautions. The abdominal skin and fur was cleaned with 70% ethanol, and after reaching the peritoneal membrane a second set of instruments sterilized with 70% ethanol was used. The



TABLE 4.1

## Constituents of Diet

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Analysis

Crude protein not less than	22.2%
Crude fat not less than	4.0%
Crude fiber not more than	5.0%
Ash not more than	3.0%

Ingredients

Ground extruded yellow corn, soybean meal, fish meal, cane molasses, wheat middlings, dehydrated alfalfa meal, ground oats, brewers' dried yeast, wheat germ meal, dried beet pulp, soybean oil, dicalcium phosphate, calcium carbonate, salt, vitamin B-12 supplement, calcium pantothenate, choline chloride, riboflavin supplement, thiamin, niacin supplement, DL methionine, vitamin A supplement, D activated animal sterol (source of vitamin D-3), vitamin E supplement, calcium iodate, manganous oxide, ferrous carbonate, cobalt carbonate, copper sulfate, zinc sulfate.

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spleen was immediately removed and transferred to a tube containing sterile culture medium. Subsequent handling of the organ was done in a Level A laminar flow hood (Enviroc, Bio-dynamics Inc., Albuquerque, New Mexico).

In experiments where kidney sections were prepared for electron microscopic examination, the kidney was the first organ removed. This was done to maintain ultrastructural integrity of the organ.

### 4.3. LYMPHOCYTE COUNTS IN BLOOD, THYMUS AND SPLEEN

#### 4.3.1. Lymphocyte count in blood

Immediately after killing the mouse, the right atrium of the heart was opened and 10  $\mu$ l of blood collected and diluted in 190  $\mu$ l of Turk's solution (0.1 g gentian violet, 1.0 ml 95% ethanol and 0.5 ml acetic acid in 98.5 ml distilled water). A small aliquot of the diluted blood was transferred to a Neubauer counting chamber, and the lymphocytes counted with a light microscope. Lymphocytes were identified by their morphological criteria and differentiated from other leukocyte types.

#### 4.3.2. Isolation and count of thymic lymphocytes

Thymus cells were obtained by gently homogenizing the organ with a tissue pestle on E-C Collector Tissue Sieve (Mandel Scientific, Rockwood, Ontario) with a mesh size 50 and opening size 0.28 mm. The contents of the gland passed through leaving the capsule behind. The product was collected in a known volume of normal saline in a petri-dish. Large debris

were broken down by gentle agitation with a Pasteur pipette and a single cell suspension was obtained by passing the material through graded needles (21G and 26G). Ten microliter aliquot of cell suspension was diluted in 190  $\mu$ l Turk's solution and the number of lymphocytes counted in Neubauer chamber. Lymphocytes were identified by their morphological criteria and differentiated from other leukocyte types. The results were expressed as total lymphocyte count in the thymus.

#### 4.3.3. Isolation and count of splenic lymphocytes

To obtain spleen cells, the organ was gently teased with two curved needles on a petri-dish containing RPMI 1640 with L-glutamine without sodium bicarbonate (No. 1060120, Flow Laboratories, McLean, Virginia) (termed RPMI 1640 henceforth). With this maneuver it was possible to squeeze out the contents from the capsule. Large cellular clumps were broken down by gentle agitation with Pasteur pipette and debris removed by sedimentation for 10 minutes. The supernatant was collected and a single cell suspension obtained by passing the material through graded needles (21G and 26G). The procedure was carried out in a known volume of culture medium. The lymphocytes were counted in Neubauer chamber and the result expressed as total count in spleen. Lymphocytes were identified by their morphological criteria and differentiated from other leukocyte types. This cell suspension was later used for other immunological assays.

In experiments where the cells were used for culture work, the entire

procedure was carried out in a laminar flow hood with total aseptic precautions.

#### 4.4. DIRECT AND INDIRECT SPLENIC PLAQUE-FORMING CELL RESPONSE

Plaque-forming cell (PFC) response in spleen was assayed by a microwell liquid matrix modification (Kappler, 1974) of the Jerne plaque-assay (Jerne, Henry, Nordin, Fuji, Koros and Lefkovits, 1974).

##### 4.4.1. Immunization of mice

Sheep red blood cells stored in Alsever solution (Woodlyn Laboratories, Guelph, Ontario) were washed three times in normal saline at 450 x g, 10 minutes each, removing the buffy coat after each wash, and resuspended to 20% (vol/vol) in normal saline. Mice were restrained and injected intraperitoneally with 0.2 ml of the red blood cell suspension. Immunization was done 4 or 8 days before killing for estimation of direct (IgM) or indirect (IgG) plaque-forming cells respectively.

##### 4.4.2. Preparation of spleen cells

Spleen cells obtained as described above (Section 4.3.3.) were washed three times in Hank's balanced salt solution (No. 18104, Flow Laboratories, McLean, Virginia) (BSS). For washing, the cell suspension was centrifuged at 200 x g for 10 minutes, supernatant aspirated and cells resuspended in the remaining media by gentle tapping. Then BSS was added to bring up the volume and the process repeated. After the final wash, cells were resuspended in BSS containing 5% (vol/vol) heat inactivated (57°C for 30

minutes) fetal calf serum, counted and adjusted to  $2 \times 10^6$  lymphocytes/ml. This stock was used for the plaque assay.

#### 4.4.3. Preparation of sheep red blood cell solution

Sheep red blood cells were washed three times in normal saline at 450 x g, 10 minutes each. The buffy coat was removed after each wash. A 15% (vol/vol) solution of sheep red blood cells was made in BSS and checked by haematocrit. Forty microliter of the solution was added to 1 ml BSS to make a 0.57% (vol/vol) solution. At this dilution, red cells were found to form a monolayer on the wells of microculture plates in the plaque assay. This stock was used as the target for lysis in the plaque assay.

#### 4.4.4. Absorption of guinea-pig complement and anti-mouse-IgG

Guinea-pig complement (Gibco Laboratories, Grand Island, New York) and rabbit anti-mouse-IgG (Miles Scientific, Rexdale, Ontario) were absorbed over sheep red blood cells to prevent non-specific binding. The sheep red cells were washed three times at 450 x g, 10 minutes each, removing the buffy coat each time. Ten milliliters guinea-pig complement or anti-mouse-IgG was then added to 2 ml packed sheep red cells and incubated for 30 minutes at 4°C with continuous rotation at 10 cycles/minute. After incubation the cells were packed by centrifugation at 450 x g for 10 minutes, supernatants removed and incubated with the sheep red blood cells again. The process was repeated three times. After the absorption, 500 µl volume of the guinea-pig complement or anti-mouse-IgG was aliquoted and stored at -70°C. For the plaque assay, 1/10 dilution

(vol/vol in BSS) of guinea-pig complement and 1/40 dilution (vol/vol in BSS) of anti-mouse-IgG was used.

#### 4.4.5. Assay for IgM antibody forming cells

The assay was performed in flat-bottom microculture plates (Flow Laboratories, McLean, Virginia). In each well of the plate, 20, 10 or 5  $\mu$ l of a suspension of 4, 2 or  $1 \times 10^6$  lymphocytes along with 50  $\mu$ l of 0.57% (vol/vol in BSS) sheep red blood cells and 50  $\mu$ l of 1/10 dilution (vol/vol in BSS) guinea pig complement were delivered. The final volume in each well was brought up to 190  $\mu$ l by adding appropriate amount of BSS. Constituents of each well are shown in Table 4.2. The assay was set up in triplicate for each sample and cell dilution. The plates were centrifuged at 55 x g for 3 minutes to obtain a monolayer and incubated for 2 hours at 37°C with 5% CO<sub>2</sub> in air. Then the number of plaques was determined with an inverted microscope. Wells for all three dilutions of lymphocytes were scanned and the triplicate samples containing the optimum number (between 15 and 25) of plaques were counted. A clear zone of red cell lysis with a lymphocyte in the centre was counted as positive (Fig. 4.1). Results are expressed as plaques/ $10^6$  spleen cells and plaques/spleen.

#### 4.4.6. Assay for IgG antibody forming cells

For the indirect plaque assay a similar protocol was followed, but an additional 50  $\mu$ l of 1/40 rabbit anti-mouse IgG added to each well (Table 4.2). Direct PFC number was also calculated and subtracted from indirect PFC number to give IgG producing cell count.

TABLE 4.2

Constituents of Culture-wells in Plaque Assay

	Lymphocytes/well			Lymphocytes/well		
	4x10 <sup>6</sup>	2x10 <sup>6</sup>	1x10 <sup>6</sup>	4x10 <sup>6</sup>	2x10 <sup>6</sup>	1x10 <sup>6</sup>
	$\mu$ l/well					
	Direct (IgM) Plaque			Indirect (IgG) Plaque		
Spleen cell suspension (2 x 10 <sup>6</sup> /ml)	20	10	5	20	10	5
Sheep Red Blood Cells (0.57%)	50	50	50	50	50	50
Guinea-pig complement (1/10)	50	50	50	50	50	50
Mouse anti-IgG (1/40)	-	-	-	50	50	50
Culture medium	70	80	85	20	30	35

## HAEMOLYTIC PLAQUES WITH ANTIBODY PRODUCING CELLS



FIG. 4.1

Two antibody producing cells. A clear area of red cell lysis surrounding each lymphocyte is counted as a plaque. (x 300).



#### 4.5. IN VITRO STIMULATION OF SPLEEN CELLS WITH MITOGENS

##### 4.5.1. Preparation of spleen cells

Spleen cell suspension was prepared as described before (Section 4.3.3.) and washed three times in RPMI 1640 (Flow Laboratories, McLean, Virginia) at  $200 \times g$  for 10 minutes each. After the final wash, cells were counted and resuspended to  $1 \times 10^6$  lymphocytes/ml in RPMI 1640 supplemented with sodium bicarbonate, 100 U/ml of penicillin, 100  $\mu\text{g/ml}$  of streptomycin, 250  $\mu\text{g/ml}$  of fungizone (all from Gibco Laboratories, Chagrin Falls, Ohio) and 5% (vol/vol) heat inactivated ( $57^\circ\text{C}$  for 30 minutes) fetal calf serum (Bocknek Organic Material, Rexdale, Ontario) (complete culture medium). Preparation of spleen cells and subsequent culture was carried out with full aseptic precautions.

##### 4.5.2. Mitogen stimulation

For setting up the stimulation culture, 200  $\mu\text{l}$  of a suspension of  $2 \times 10^5$  lymphocytes and an optimum amount of mitogen [18  $\mu\text{g}$  of phytohaemagglutinin (PHA) (Wellcome Diagnostics, Dartford, England) or 1  $\mu\text{g}$  concanavalin A (Con A) (Sigma Chemical Company, St. Louis, Missouri)] diluted in 20  $\mu\text{l}$  of normal saline was delivered into each well of a flat-bottom microculture plate (Flow Laboratories, McLean, Virginia). The optimum amount of mitogen was established in a preliminary dose-response titration and the same stock was used throughout the experiment. In control cultures, 20  $\mu\text{l}$  of saline was added in place of mitogen. Cultures were set up in triplicate for each mitogen and sample. The culture plates were then incubated for 72 hours at  $37^\circ\text{C}$  in a humidified atmosphere.

containing 5% CO<sub>2</sub> in air. To assess DNA synthesis, 0.5  $\mu$ Ci of [<sup>3</sup>H]thymidine with a specific activity of 2 Ci/mM (New England Nuclear, Boston, Massachusetts) was added to each well for the final 4 hours of incubation. Cells were then harvested on glass fiber filters (Mandel Scientific, Rockwood, Ontario) with a multiple automated cell harvester (Titertek, Flow Laboratories, Rockville, Maryland). The filter papers were dried and discs put in scintillation vials with 8 ml of liquid scintillation solution (BDH Chemicals, Toronto, Ontario) and counted for radioactivity by liquid scintillation spectrophotometry (Beckman Instruments LS-330 Liquid Scintillation System, Fullerton, California). The results were expressed as stimulation index as follows :

$$\text{Stimulation index} = \frac{\text{mean counts per minute of triplicate stimulated culture}}{\text{Mean counts per minute of triplicate control culture}}$$

#### 4.6. ASSAY OF SUPPRESSOR CELL ACTIVITY

Suppressor cell activity was assayed by coculturing concanavalin A (Con A) stimulated spleen cells (suppressor cell) with autologous spleen cells (responder cell) and measuring the mitogenic response of the responder cell to phytohaemagglutinin (PHA) (Catalona, Ratliff and McCool 1980; Grzelak, Olszewski and Engeset, 1983). Asepsis was maintained in all stages of the assay.

#### 4.6.1. Activation of suppressor cells

Spleen cells were prepared as described before (Section 4.5.1.) and resuspended to  $2 \times 10^6$  lymphocytes/ml in complete culture medium. Three milliliters of the suspension ( $6 \times 10^6$  lymphocytes) was incubated with 60  $\mu$ l of 0.5 mg/ml (10  $\mu$ g/ml final dilution) Con A (Sigma Chemical Company, St. Louis, Missouri) for 18 hours at 37°C in an atmosphere containing 5% CO<sub>2</sub> in air. After incubation, cells were washed twice at 250 x g for 15 minutes each and treated with 100  $\mu$ l/ml mitomycin C (Sigma Chemical Company, St. Louis, Missouri) for 1 hour at 37°C to stop further multiplication. The cells were then washed twice with 0.3 M. methyl- $\alpha$ -mannopyranoside (Sigma Chemical Company, St. Louis, Missouri) and thrice with RPMI 1640 at 250 x g for 15 minutes each and resuspended to  $1 \times 10^6$  lymphocytes/ml in complete culture medium. Cells thus activated were used as suppressor cells (SC) in the assay.

Another batch of cells were treated identically except that they were not stimulated with Con A. However, Con A was added to the unstimulated cells after the 18 hour incubation to control for the effects of the Con A itself being carried over with the cells in the subsequent steps. These cells were used as controls and are called natural suppressor cells (NSC).

#### 4.6.2. Responder cells

Autologous spleen cells (prepared as described in Section 4.5.1.) suspended to  $1 \times 10^6$  lymphocytes/ml in complete culture medium were used as responder cells (RC) in the coculture.

#### 4.6.3. Suppressor cell activity

In each well of a flat-bottom microculture plate (Flow Laboratories, McLean, Virginia) 100  $\mu$ l (containing  $1 \times 10^5$  lymphocytes) of RC ~~and~~ 100  $\mu$ l (containing  $1 \times 10^5$  lymphocytes) of SC or NSC was added and cultured with or without 18  $\mu$ g/well PHA (Wellcome Diagnostics, Dartford, England) for 72 hours at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. Triplicate culture was set up for each of the four combinations (RC + SC + PHA, RC + SC - PHA, RC + NSC + PHA and RC + NSC - PHA) and samples. To assess DNA synthesis, 0.5  $\mu$ Ci of [<sup>3</sup>H]thymidine with a specific activity of 2 Ci/mM (New England Nuclear, Boston, Massachusetts) was added to each well for the final 4 hours of incubation. Cells were then harvested on glass fiber filters (Mandel Scientific, Rockwood, Ontario) with a multiple automated cell harvester (Titertek, Flow Laboratories, Rockville, Maryland). The filter papers were dried and discs put in scintillation vials with 8 ml of liquid scintillation solution (BDH Chemicals, Toronto, Ontario) and counted for radioactivity by liquid scintillation spectrophotometry (Beckman Instruments LS-330 liquid scintillation system, Fullerton, California). Suppressor cell activity (SCA) was calculated using the mean counts per minute of the triplicate cultures and expressed as a percentage as follows:

$$SCA = \left| 1 - \frac{(RC + SC + PHA) - (RC + SC - PHA)}{(RC + NSC + PHA) - (RC + NSC - PHA)} \right| \times 100$$

#### 4.7. ENUMERATION OF B LYMPHOCYTES AND T LYMPHOCYTE SUBSETS IN SPLEEN

Enumeration of B lymphocytes and T lymphocyte subsets in the spleen was done by immunofluorescence staining using appropriate specific polyclonal or monoclonal antibodies. The antibodies used were fluorescein isothiocyanate (FITC)-conjugated affinity purified goat anti-mouse Ig for staining B cells, and purified monoclonal anti-L3T4 and FITC-conjugated monoclonal anti-Lyt-2 for the T cell subsets (all from Becton Dickinson Immunocytometry Systems, Mountain View, California). Anti-L3T4 and anti-Lyt-2 identify the murine differentiation glycoproteins CD4 and CD8 which are markers of helper/inducer and suppressor/cytotoxic T cells respectively (Adkins, Mueller, Okada, Reichert, Weissman and Spangrude, 1987).

B cells and Lyt-2+ cells were stained by direct immunofluorescence technique and L3T4+ cells by indirect technique (Mishell and Shiigi, 1980).

##### 4.7.1. Direct immunofluorescence staining

Spleen cell suspension was prepared as described before (Section 4.3.3.), washed thrice at 200 x g for 10 minutes each and adjusted to  $2 \times 10^7$  cells/ml in RPMI 1640. Fifty microliters ( $1 \times 10^6$  cells) of the stock was incubated with 1  $\mu$ g of FITC conjugated anti-Lyt-2 or goat anti-mouse Ig for 30 minutes at room temperature in dark. After incubation, the cells were washed twice with Dulbecco-phosphate-buffered saline (PBS) (No. 450-1300, Gibco Laboratories, Grand Island, New York) containing 0.1% (vol/vol) sodium azide at 200 x g for 5 minutes each and resuspended in a drop of mounting medium (10% glycerol in PBS). A drop of the cell

suspension was placed on a glass slide, coded and the number of fluorescent and total cells were counted with a Leitz fluorescent microscope (Ernst Leitz Wetzlar, West Germany). Monocytes were excluded by morphological criteria with phase contrast microscopy. One hundred fluorescent cells were counted and the results expressed as a percentage of total cells.

#### 4.7.2. Indirect immunofluorescence staining

The spleen cells were first tagged with purified anti-L3T4 and washed as for direct staining method. Then the cells were incubated with 1  $\mu$ g of phycoerythrin-conjugated rat anti-mouse Kappa light-chain antibody (Becton Dickinson Immunocytometry Systems, Mountain View, California) for 30 minutes at room temperature. After incubation, the cells were washed, resuspended in mounting medium and counted as for the direct staining method.

#### 4.8. NATURAL KILLER CELL ACTIVITY

Natural killer cell activity of splenic lymphocytes were performed by a microwell modification of the chromium release assay described by Kiessling, Klein and Wigzell (1975), and Kiessling, Klein, Pross and Wigzell (1975).

##### 4.8.1. Preparation of splenic lymphocytes.

Spleen cells were obtained as described above (Section 4.3.3.) and the lymphocytes isolated by density gradient centrifugation. The suspension of

spleen cells was layered over Ficoll-Hypaque (Sigma Chemical Company, St. Louis, Missouri) with a specific gravity of 1.09 and centrifuged at  $400 \times g$  for 30 minutes at room temperature. The lymphocytes which formed a visible interface were collected, washed three times in RPMI-1640 at  $200 \times g$  for 10 minutes each, counted and adjusted to a concentration of  $20 \times 10^6$  cells/ml in RPMI-1640 supplemented with sodium bicarbonate, 100 U/ml of penicillin, 100  $\mu\text{g/ml}$  of streptomycin, 250  $\mu\text{g/ml}$  of fungizone (all from Gibco Laboratories, Chagrin Falls, Ohio) and 10% (vol/vol) heat inactivated ( $57^\circ\text{C}$  for 30 minutes) fetal calf serum (Bocknek Organic Material, Rexdale, Ontario) (complete culture medium). This stock was used as the effector cells in the natural killer cell assay.

#### 4.8.2. Labelling of target cells

Moloney virus induced mouse lymphoma cell line YAC-1 (American Type Tissue Culture, Rockville, Maryland) maintained in continuous culture in complete culture medium was used as target cell. For labelling, 200  $\mu\text{Ci}$   $^{51}\text{Cr}$  as sodium chromate was added to  $5 \times 10^6$  tumour cells in 0.5 ml of culture medium and incubated for 60 minutes at  $37^\circ\text{C}$  with continuous rotation at 10 cycles/minute. The cells were then washed twice at  $200 \times g$  for 10 minutes each at room temperature, counted, and resuspended to the final concentration of  $0.8 \times 10^6$  cells/ml.

#### 4.8.3. Chromium release assay

For setting up the assay, 50  $\mu\text{l}$  labelled target cell suspension ( $4 \times 10^4$  cells) and 100  $\mu\text{l}$  effector cell suspension ( $2 \times 10^6$  cells) was delivered into

each well of a V-bottom microculture plate (Flow Laboratories, McLean, Virginia). This corresponded to an effector target ratio of 50:1. Spontaneous  $^{51}\text{Cr}$  release from target cells was measured in the absence of effector cells, and the total release was determined by treating the target cells with 100  $\mu\text{l}$  of 1% (weight/volume) Nonidet P40 (Sigma Chemical Company, St. Louis, Missouri). The volume in each well was maintained at 200  $\mu\text{l}$  by adding appropriate amount of culture medium. Final constituents of the culture wells are shown in Table 4.3. The plates were then incubated at 37°C in 5%  $\text{CO}_2$  in air for 4 and 12 hours. Two sets of culture, each in quadruplicate, were set up for the two time periods. After incubation, the plates were centrifuged at 400 x g for 5 minutes and 100  $\mu\text{l}$  of supernatant was collected from each well and counted for radioactivity in a Beckman 310 system gamma counter (Beckman Instruments, Fullerton, California). The results were expressed as percentage lysis as follows :

$$\% \text{ lysis} = \frac{\begin{array}{l} \text{mean counts per minute} \\ \text{of experimental release} \end{array} - \begin{array}{l} \text{mean counts per minute} \\ \text{of spontaneous release} \end{array}}{\begin{array}{l} \text{mean counts per minute} \\ \text{of total release} \end{array} - \begin{array}{l} \text{mean counts per minute} \\ \text{of spontaneous release} \end{array}} \times 100$$

Spontaneous release was less than 10 percent of total release in 4 hour culture and varied between 20 and 30 percent in 12 hour culture.



TABLE 4.3

Constituents of Culture Wells in Natural-killer Cell Assay

	50:1 target/effector ratio	Total release	Spontaneous release
	$\mu\text{l/well}$	$\mu\text{l/well}$	$\mu\text{l/well}$
Effector cell ( $20 \times 10^6/\text{ml}$ )	100	-	-
Target cell ( $0.8 \times 10^6/\text{ml}$ )	50	50	50
Culture medium	50	50	150
1-0% Nonidet P40	-	100	-

#### 4.9. POKEWEED MITOGEN STIMULATED IgG PRODUCTION

##### 4.9.1. Pokeweed mitogen stimulation of lymphocytes

Lymphocytes were separated by density gradient centrifugation as described before (Section 4.8.1.) and resuspended to  $1 \times 10^6$  cells/ml in complete culture medium. Two milliliters of the stock ( $2 \times 10^6$  cells) was incubated with 200  $\mu$ l of 1/20 dilution of pokeweed mitogen (Gibco Laboratories, Chagrin Falls, Ohio) for 7 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. The cells were then packed by centrifugation at 450 x g for 10 minutes and the supernatants collected and stored at -20°C. The samples were later tested for IgG level by enzyme linked immunosorbent assay. None of the samples were stored for longer than 3 months when tested for IgG level.

##### 4.9.2. Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) was based on the method described by Voller and Bidwell (1986). For reading, calculation and expression of ELISA data a modification of the computer assisted method described by Ritchie, Nickerson and Fuller (1981) was used.

Optimum concentrations of reagents and analytes were first determined by standard titrations. Then the samples were assayed in one batch. The analytes were measured over the concentration range in which the ELISA had steep dose-response characteristics.

Individual wells of a 96-well polystyrene E.I.A. microtitration plates (No. 7638104, Flow Laboratories, McLean, Virginia) were first sensitized by

passive absorption with anti-mouse IgG. For the coating, 200  $\mu$ l of affinity purified goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania) diluted 1/500 (vol/vol in bicarbonate buffer, pH 9.6) was added to each well of the plate and incubated overnight (16 hours) at 4°C. The plates were then washed once with 200  $\mu$ l phosphate-buffered saline containing 0.05% (vol/vol) Tween-20 (PBS-T) (Sigma Chemical Company, St. Louis, Missouri). For washing, the contents of the wells were flicked off and the plate dried on a tissue paper, then PBS-T was added to the wells and the contents flicked off and the plates dried again. Tween-20 (polyoxyethylene sorbitan monolaurate) is a wetting agent and prevents non-specific binding of immunoglobulins to the plastic surface. The uncoated surface of the wells was further blocked by adding 200  $\mu$ l of blocking buffer (0.1 ml/ml heat inactivated goat serum, 0.01 gm/ml bovine serum albumin and 0.003 gm/ml gelatin in PBS) to each well for 1 hour at room temperature. The plates were then washed once with PBS-T. The first well in each row was not coated to get the background reading of the plate.

Chromatographically purified mouse IgG (No. 7176, Jackson Immunoresearch Laboratories, West Grove, Pennsylvania) was used as the standard. Serial dilutions of the standard were made in PBS-T to give concentrations of 400, 200, 100, 50, 12.5, 6.25 and 3.125 ng IgG/ml. In different wells of the coated E.I.A. plates, 200  $\mu$ l of the standards or supernatant samples were added and incubated overnight (16 hours) at 4°C. Each standard and sample was run in quadruplicate. The plates were then washed four times with PBS-T to remove unreacted immunoglobulins. Subsequently 200  $\mu$ l of 1/100,000 dilution (vol/vol in PBS, containing 5%

goat serum) affinity purified goat anti-mouse IgG conjugated with the enzyme horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania) was added to each well and incubated at room temperature for 2 hours. This conjugate reacts with the antibody captured by the sensitized plate. After incubation, the plates were washed six times with PBS-T to remove unreactive conjugate. Then 200  $\mu$ l freshly prepared orthophenylenediamine [0.4 mg/ml in phosphate citrate buffer (pH 5) containing 0.4  $\mu$ l 30%  $\text{H}_2\text{O}_2$ /ml] was added to each well and the plate incubated at room temperature in the dark. The substrate reacts with the enzyme to produce a yellow coloured product. The colour reaction was stopped at 20 minutes by adding 50  $\mu$ l of 2.5 N  $\text{H}_2\text{SO}_4$  to each well. Acid treatment further changed the colour to a stable orange-brown product which was then read photoelectrically by a Titertek Multiscan ELISA reader (Eflab Oy, Helsinki, Finland) with wave length set at 492 nm. The reading was given as optical density.

The concentrations of samples were calculated from the standard curve (Fig. 4.2) using a computer program run in the Memorial University mainframe VAX/VMS computer. The program subtracts the background readings from test readings and uses this corrected optical density in calculations. Absorbances that fall within 5 to 95 percent of the maximum observed absorbance are only included in the analyses. The results were expressed as ng/ml.

## Standard Curve for ELISA

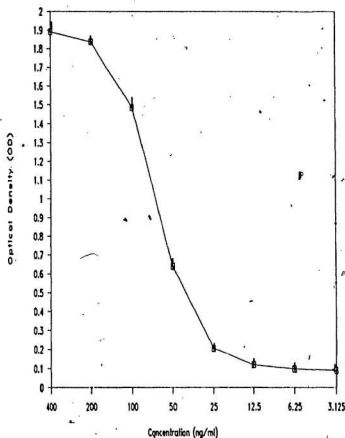


FIG. 4.2

Curve showing the absorbance value for serial two fold dilutions of IgG standard. Data are given as means  $\pm$  SD.

#### 4.10. AUTOIMMUNE RESPONSE IN KIDNEY

##### 4.10.1. Preparation of kidney sections

Kidneys were placed on blocks and covered with OCT embedding compound (Miles Scientific, Naperville, Illinois). The block was placed in petroleum ether for 5 minutes which was cooled with CO<sub>2</sub> in acetone. The frozen tissues were sectioned to a thickness of 5  $\mu$ m in a cryostat and mounted on a glass slide. Kidney sections were prepared on the day of killing. The sections were then stored at -20°C and examined later on the same week.

##### 4.10.2. Staining and examination of the sections

The sections were incubated at room temperature with 1/20 dilution (vol/vol) of fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG or complement C3 (Cappel, Division of Cooper Diagnostic, Cochranville, Pennsylvania) for 30 minutes, rinsed in a PBS bath for 3 hours, changing the PBS every 30 minutes, dried and examined with fluorescent light microscope for deposition of antibodies and complement.

#### 4.11. ELECTRON MICROSCOPY OF KIDNEY

Kidney sections were examined by transmission electron microscopy at 0 and 6 weeks of observation. At least four animals from each group were examined. The procedure was performed by the Electron Microscopy Department, Faculty of Medicine, Memorial University of Newfoundland.

Kidneys were collected immediately after killing the animal and processed for electron microscopic examination. The tissue was diced into small pieces (1 cubic mm approx.) and immersed for 20 minutes in a mixture of paraformaldehyde and glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.4) for fixation (Karnovsky, 1965). After fixation the tissues were washed thoroughly with 0.1M sodium cacodylate buffer and postfixed for 10 minutes in 1% (vol/vol) osmium tetroxide in 0.2M sodium cacodylate buffer. Then they were washed with 0.1M sodium cacodylate buffer and dehydrated by passing through serial concentrations (70%, 95% and 100%) of ethanol. The exposure was for 3 minutes in each concentration with two changes, and 10 minutes for absolute ethanol with two changes. After dehydration, the tissues were immersed in absolute acetone for 10 minutes with two changes, and then transferred to a 50:50 (vol:vol) mixture of absolute acetone and Epon 812 embedding resin for 10 minutes, and finally to 100% resin for 10 minutes with two changes. All these steps were carried out in a fume hood at room temperature and the tissues were placed on a rotator. Then the tissues were embedded with resin in capsules and polymerized at 70°C for 16 hours in a Reichert KT-100 oven.

Subsequently 0.5  $\mu$ m sections were cut with a LKB Huxley ultramicrotome and ultra-thin silver sections were prepared with a Reichert OMU3 ultratome. The sections were poststained with lead citrate and uranyl acetate and observed under a Phillips 300 transmission electron microscope.

Sections 0.5  $\mu$ m thick, stained with 1% toluidine blue in 1% sodium borate were also observed under a light microscope.

#### 4.12. TRACE ELEMENT ANALYSIS

Portions of liver and kidney were weighed, freeze dried for 24 hours and stored at 4°C for cadmium analyses. The analyses were done in the laboratory of Dr. James K. Friel, Department of Biochemistry, Memorial University of Newfoundland. The tissues were ashed at 500°C for 16 hours in porcelain crucibles. The ash was dissolved for 30 minutes in 0.5 ml pure  $\text{HNO}_3$  and 4.5 ml 1 N HCl and the resulting solution was made up to 10 ml with deionized distilled water. Acid washed containers were used in all stages of the procedure. The samples were analyzed by atomic absorption spectroscopy (Perkin Elmer, Norwalk, Connecticut) (Beaty, 1978). Cadmium in the control mice tissues were analyzed with a HGA-300 graphite atomizer with deuterium arc background correction, and the remaining samples were analyzed by flame with a single slot 4-inch burner head. Standards (Bovine Liver, No. 1577a, National Bureau of Standards, Gaithersburg, Maryland) were also analyzed simultaneously to assure accuracy of the assay. All results are reported as  $\mu\text{g/g}$  wet weight of organ analyzed.

A second batch of liver samples (Experiment 4) were analyzed for multiple trace elements through the courtesy of Dr. Victor Conti, Lifeline Clinical Laboratory, BALCO, San Mateo, California. The analysis was done by 'Inductively Coupled Plasma Spectrophotometry'. In this procedure, the samples were prepared by ashing in perchloric acid:nitric acid and the volume made up in deionized distilled water. The samples were injected into an argon stream of plasma, heated to 10,000 K by induction coils, and



the elements quantitated simultaneously on the basis of induced atomic emission. Results are expressed as  $\mu\text{g/g}$  of wet weight of tissue.

#### 4.13. DATA HANDLING AND STATISTICAL ANALYSIS

Data were stored on EDT file in the VAX/VMS mainframe computer system of the Memorial University of Newfoundland (EDT Editor Manual, 1980) and analyzed by the statistical packages MINITAB (Ryan, Joiner and Ryan, 1985a; Ryan, Joiner and Ryan, 1985b) and SAS (Cody and Smith, 1985). Data were stored in a two-dimensional matrix suitable for handling by the statistical packages. A one-way analysis of variance was performed to compare the groups. When difference was found to be present at  $P < 0.05$ , treatment effect was inferred and Duncan's multiple-range test was performed to make multiple comparison between the means. Significance levels used were 0.05 or less. When analysis of variance did not reveal any significant difference, no further analysis was done. All results are reported as mean  $\pm$  SD.

## CHAPTER 5

### RESULTS

#### 5.1. EXPERIMENT 1

##### 5.1.1. General health of mice

Mice were examined at least once a week for obvious manifestations of toxicity, failure to gain weight, altered food intake and death. No outward manifestations of any toxic effect was seen in any animal and none of the animals died on the treatment schedules. Also on dissection after killing no gross pathological lesions were seen in any organs. The animals thus appeared to tolerate the treatment well.

Weights of the animals measured weekly are shown for four time periods in Table 5.1. There were no statistically significant differences between the treatment groups, also the weight gain was similar in all groups.

Food disappearance data are shown in Table 5.2. Food pellets were weighed weekly at the same time as the animals and the results were averaged for the preceding 3 weeks. The rather large amount of food pellets disappearing was due to the nature of housing. In wire-meshed hanging cages, a considerable amount of food is wasted by the animals, since pellets of smaller size tend to drop off. So the measurements

TABLE 5.1

Weight of Mice

Treatment	Group	Before treatment	0 week	3 week	6 week
gm					
None	I	18.6 $\pm$ 1.4	20.6 $\pm$ 1.4	22.8 $\pm$ 1.3	23.9 $\pm$ 1.8
Cd for 3 wk and none for next 3 or 6 wk	II	18.7 $\pm$ 1.4	21.0 $\pm$ 1.4	23.3 $\pm$ 1.7	25.0 $\pm$ 1.6
Cd with Zn for 3 wk and Zn for next 3 or 6 wk	III	18.8 $\pm$ 1.8	21.6 $\pm$ 1.6	23.9 $\pm$ 1.7	25.4 $\pm$ 1.7
Cd for 3 wk and Zn for next 3 or 6 wk	IV	18.2 $\pm$ 1.4	20.8 $\pm$ 1.9	23.6 $\pm$ 1.8	25.1 $\pm$ 1.7
Zn for 3, 6 or 9 wk	V	18.6 $\pm$ 1.3	21.0 $\pm$ 1.6	23.7 $\pm$ 1.3	24.7 $\pm$ 1.2
None for 3 wk and Zn for next 3 or 6 wk	VI	18.1 $\pm$ 1.3	20.9 $\pm$ 1.5	23.2 $\pm$ 1.3	24.7 $\pm$ 1.5

Results are expressed as mean  $\pm$  SD. Before treatment and at 0 week, there were 30 animals in Groups I, II, III and V, and 20 in Groups IV and VI. At 3 and 6 week, there were 20 and 10 animals in each group.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

TABLE 5.2

## Food Disappearance

Treatment	Group	0 week	3 week	6 week
gm/day				
None	I	6.5 $\pm$ 1.2	6.9 $\pm$ 1.1	6.1 $\pm$ 1.5
Cd for 3 wk and none for next 3 or 6 wk	II	6.1 $\pm$ 0.8	6.8 $\pm$ 1.3	6.3 $\pm$ 1.2
Cd with Zn for 3 wk and Zn for next 3 or 6 wk	III	6.3 $\pm$ 1.0	6.4 $\pm$ 1.0	6.5 $\pm$ 0.9
Cd for 3 wk and Zn for next 3 or 6 wk	IV	6.4 $\pm$ 0.9	6.6 $\pm$ 0.8	6.4 $\pm$ 1.4
Zn for 3, 6 or 9 wk	V	6.0 $\pm$ 1.3	6.7 $\pm$ 1.2	6.2 $\pm$ 1.2
None for 3 wk and Zn for next 3 or 6 wk	VI	6.6 $\pm$ 1.5	6.4 $\pm$ 0.8	6.3 $\pm$ 1.1

Food disappearance of individually-caged mice were measured weekly.

Results are expressed as mean  $\pm$  SD food disappearance for the preceding 3 weeks. At 0 week, there were 30 animals in Groups I, II, III and V, and 20 in Groups IV and VI. At 3 and 6 week, there were 20 and 10 animals in each group.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

presented in Table 5.2 reflect the efforts of mice on food procurement, rather than true food consumption. The results did not differ between the six treatment groups and three observations periods.

#### 5.1.2. Weight of different organs

Liver, kidneys, thymus and spleen from all animals were weighed immediately after killing. Results are shown in Tables 5.3, 5.4, 5.5 and 5.6. There were no significant differences between the treatment groups at any observation times, although the liver and kidneys tended to be heavier at the 6 week observation period (i.e. 6 weeks after cessation of cadmium treatment) in animals receiving cadmium alone or with zinc as compared to animals receiving no treatment or zinc (Tables 5.3 and 5.4). However, the difference did not reach the level of statistical significance. The thymus tended to decrease in weight with increasing age of mice (Table 5.5).

#### 5.1.3. Lymphocyte counts in blood, thymus and spleen

Lymphocytes were counted in blood collected from the right atrium of the heart and in cell suspension prepared from homogenized thymus and spleen. The results are shown in Tables 5.7, 5.8 and 5.9. The counts did not differ between the treatment groups at any observation time. However, the lymphocyte content of thymus tended to decrease with increasing age of mice and that of spleen tended to increase. Thus cadmium and zinc, at the doses used in this experiment did not alter the total lymphocyte content in these organs.

TABLE 5.3

## Weight of Liver

Treatment	0 week	3 week	6 week
	mg		
None	1333.6 $\pm$ 160.8	1396.0 $\pm$ 149.3	1385.5 $\pm$ 108.1
Cd for 3 wk and none for next 3 or 6 wk	1416.8 $\pm$ 163.1	1283.1 $\pm$ 231.7	1497.2 $\pm$ 160.7
Cd with Zn for 3 wk and Zn for next 3 or 6 wk	1383.6 $\pm$ 117.8	1380.7 $\pm$ 190.7	1573.6 $\pm$ 160.5
Cd for 3 wk and Zn for next 3 or 6 wk	-	1341.7 $\pm$ 187.9	1551.0 $\pm$ 192.7
Zn for 3, 6 or 9 wk	1403.8 $\pm$ 132.4	1487.3 $\pm$ 97.6	1404.4 $\pm$ 156.7
None for 3 wk and Zn for next 3 or 6 wk	-	1430.0 $\pm$ 125.8	1432.5 $\pm$ 174.7

Values are expressed as mean  $\pm$  SD of 10 observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

TABLE 5.4

## Weight of Kidneys

Treatment	0 week	3 week	6 week
	-mg		
None	286.3 $\pm$ 36.0	339.9 $\pm$ 20.0	354.2 $\pm$ 22.0
Cd for 3 wk and none for next 3 or 6 wk	312.9 $\pm$ 37.2	326.8 $\pm$ 35.6	396.9 $\pm$ 68.6
Cd with Zn for 3 wk and Zn for next 3 or 6 wk	297.1 $\pm$ 25.8	343.6 $\pm$ 28.8	389.7 $\pm$ 35.5
Cd for 3 wk and Zn for next 3 or 6 wk	-	340.9 $\pm$ 42.5	387.6 $\pm$ 31.0
Zn for 3, 6 or 9 wk	305.4 $\pm$ 30.5	356.2 $\pm$ 34.8	374.1 $\pm$ 44.0
None for 3 wk and Zn for next 3 or 6 wk	-	335.5 $\pm$ 29.4	367.5 $\pm$ 42.6

Values are expressed as mean  $\pm$  SD of 10 observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

TABLE 5.5

## Weight of Thymus

Treatment	0 week	3 week	6 week
	mg		
None	48.5 $\pm$ 4.8	43.5 $\pm$ 12.3	35.7 $\pm$ 9.6
Cd for 3 wk and none for next 3 or 6 wk	52.4 $\pm$ 17.1	39.3 $\pm$ 13.2	39.5 $\pm$ 7.2
Cd with Zn for 3 wk and Zn for next 3 or 6 wk	45.5 $\pm$ 8.5	39.7 $\pm$ 5.6	39.7 $\pm$ 4.2
Cd for 3 wk and Zn for next 3 or 6 wk		46.4 $\pm$ 11.5	35.5 $\pm$ 5.2
Zn for 3, 6 or 9 wk	49.7 $\pm$ 8.3	42.8 $\pm$ 5.4	39.2 $\pm$ 4.6
None for 3 wk and Zn for next 3 or 6 wk		45.3 $\pm$ 4.7	36.3 $\pm$ 2.7

Values are expressed as mean  $\pm$  SD of 10 observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.



TABLE 5.6

## Weight of Spleen

Treatment	0 week	3 week	6 week
	mg		
None	106.2 $\pm$ 20.2	99.2 $\pm$ 12.4	93.4 $\pm$ 9.0
Cd for 3 wk and none for next 3 or 6 wk	112.6 $\pm$ 42.8	103.0 $\pm$ 21.8	107.2 $\pm$ 8.7
Cd with Zn for 3 wk and Zn for next 3 or 6 wk	97.0 $\pm$ 9.6	99.9 $\pm$ 8.6	107.8 $\pm$ 14.4
Cd for 3 wk and Zn for next 3 or 6 wk	-	103.6 $\pm$ 16.3	106.6 $\pm$ 12.7
Zn for 3, 6 or 9 wk	92.5 $\pm$ 11.0	108.1 $\pm$ 17.3	109.8 $\pm$ 17.2
None for 3 wk and Zn for next 3 or 6 wk	-	100.1 $\pm$ 10.3	108.8 $\pm$ 22.2

Values are expressed as mean  $\pm$  SD of 10 observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

TABLE 5.7

## Lymphocyte Counts in Blood

Treatment	0 week	3 week	6 week
		$\times 10^6/\text{ml}$	
None	$2.25 \pm 0.91$	$2.99 \pm 0.76$	$2.45 \pm 0.70$
Cd for 3 wk and none for next 3 or 6 wk	$2.90 \pm 1.21$	$3.01 \pm 0.83$	$2.59 \pm 0.51$
Cd with Zn for 3 wk and Zn for next 3 or 6 wk	$3.02 \pm 1.17$	$2.81 \pm 0.82$	$3.22 \pm 0.77$
Cd for 3 wk and Zn for next 3 or 6 wk	-	$2.83 \pm 1.17$	$2.45 \pm 0.78$
Zn for 3, 6 or 9 wk	$2.52 \pm 1.06$	$2.32 \pm 1.04$	$2.93 \pm 0.66$
None for 3 wk and Zn for next 3 or 6 wk	-	$2.43 \pm 0.90$	$2.85 \pm 0.79$

Values are expressed as mean  $\pm$  SD of 10 observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

TABLE 5.8

## Lymphocyte Counts in Thymus

Treatment	0 week	3 week	6 week
	$\times 10^6$		
None	96.6 $\pm$ 43.4	110.7 $\pm$ 35.3	81.0 $\pm$ 32.1
Cd for 3 wk and none for next 3 or 6 wk	104.3 $\pm$ 63.8	82.6 $\pm$ 51.8	92.5 $\pm$ 17.9
Cd with Zn for 3 wk and Zn for next 3 or 6 wk	87.4 $\pm$ 46.7	83.2 $\pm$ 26.9	80.0 $\pm$ 12.8
Cd for 3 wk and Zn for next 3 or 6 wk		104.8 $\pm$ 46.0	77.5 $\pm$ 17.3
Zn for 3, 6 or 9 wk	118.6 $\pm$ 53.2	99.0 $\pm$ 33.7	75.8 $\pm$ 18.7
None for 3 wk and Zn for next 3 or 6 wk		96.1 $\pm$ 30.3	79.7 $\pm$ 13.6

Values are expressed as mean  $\pm$  SD of 10 observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

TABLE 5.9

## Lymphocyte Counts in Spleen

Treatment	0 week	3 week	6 week
		$\times 10^6$	
None	198.1 $\pm$ 53.2	205.3 $\pm$ 31.7	216.1 $\pm$ 16.6
Cd for 3 wk and none for next 3 or 6 wk	192.9 $\pm$ 96.1	216.1 $\pm$ 48.1	224.3 $\pm$ 24.6
Cd with Zn for 3 wk and Zn for next 3 or 6 wk	174.7 $\pm$ 48.0	215.4 $\pm$ 28.5	219.8 $\pm$ 28.9
Cd for 3 wk and Zn for next 3 or 6 wk	-	200.5 $\pm$ 31.7	222.7 $\pm$ 35.1
Zn for 3, 6 or 9 wk	158.0 $\pm$ 24.6	212.9 $\pm$ 37.7	222.3 $\pm$ 25.4
None for 3 wk and Zn for next 3 or 6 wk	-	212.5 $\pm$ 36.4	219.8 $\pm$ 29.7

Values are expressed as mean  $\pm$  SD of 10 observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

#### 5.1.4. Direct plaque-forming cell response

The direct plaque-forming cell responses of all mice were assayed by injecting sheep red blood cells (SRBC) 4 days prior to killing and counting the number of splenic lymphocytes producing IgM antibody against the SRBC antigen in a plaque-assay. Results expressed as plaques/ $10^6$  spleen lymphocytes and plaques/spleen are shown in Fig. 5.1 and Table 5.10 respectively.

Mice treated with cadmium for 3 weeks (week 0 observation) had significantly increased number of splenic IgM plaque-forming cell (PFC) as compared to those receiving cadmium with zinc, zinc or no treatment. Thus concurrent administration of zinc prevented the cadmium-induced increase in IgM PFC response. Three weeks after cessation of cadmium treatment (week -3 observation), the number of IgM antibody-forming cells still tended to be high in cadmium-treated animals, and the antagonistic effect of zinc on cadmium was still maintained, although the differences were statistically not significant. Even zinc given after cessation of cadmium treatment tended to lower the cadmium-induced increase in IgM PFC number (Fig. 5.1 and Table 5.10). After 6 weeks of cessation of cadmium treatment (week 6 observation) no significant difference in IgM antibody-forming cells between the groups were observed (Table 5.10).

#### 5.1.5. Proliferative response of spleen cells

Spleen cell cultures were stimulated with the T cell mitogens PHA and Con A for 72 hours and radioactive thymidine incorporation during the last 4 hours of the culture was measured. The ratio between the thymidine

## IgM PLAQUES

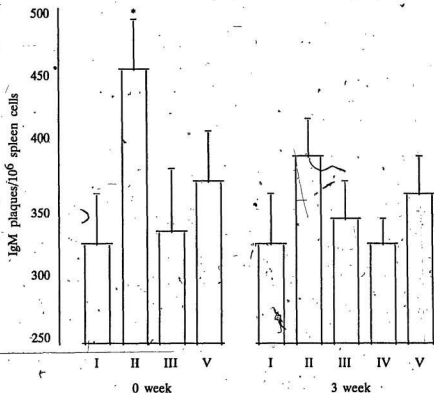


FIG. 5.1

Direct plaque-forming (IgM) cells response in mice treated with cadmium with or without zinc. Group I received no treatment; group II, cadmium for 3 weeks and no treatment for next 3 weeks; group III, cadmium with zinc for 3 weeks and zinc for next 3 weeks; group IV, cadmium for 3 weeks and zinc for next 3 weeks; and group V, zinc for 6 weeks. Bars represent mean and SD of 10 observations. At 0 week, cadmium treated animals had higher number of plaque-forming cells than the other three groups ( $P < 0.05$ ). Simultaneous treatment of cadmium and zinc did not result in a greater number of plaque-forming cells. At 3 week, there were no significant differences between the groups.

TABLE 5.10

## Direct Plaque-forming Cell Response

Treatment	0 week	3 week	6 week
IgM Plaques/spleen			
None	63364 $\pm$ 11235	67125 $\pm$ 14262	75432 $\pm$ 10494
Cd for 3 wk and none for next 3 or 6 wk	89466 $\pm$ 13104*	81793 $\pm$ 10331	81432 $\pm$ 12923
Cd with Zn for 3 wk and Zn for next 3 or 6 wk	56683 $\pm$ 11147	71958 $\pm$ 10026	77450 $\pm$ 11428*
Cd for 3 wk and Zn for next 3 or 6 wk	-	67315 $\pm$ 13803	77728 $\pm$ 11980
Zn for 3, 6 or 9 wk	57276 $\pm$ 12161	68483 $\pm$ 9206	79912 $\pm$ 12498
None for 3 wk and Zn for next 3 or 6 wk	-	68380 $\pm$ 12222	76137 $\pm$ 10424

Values are expressed as mean  $\pm$  SD of 10 observations.

\*Significantly different at  $P < 0.05$  from other values in the same column by Duncan's multiple-range test.

uptake of stimulated and non-stimulated cells was expressed as stimulation index. Results are shown in Table 5.11. There was a large spread of values obtained from the different groups. Differences between the groups were not statistically significant, however, the mean stimulation index tended to be higher in animals receiving cadmium as compared to the non-treated controls, both at 0 and 3 week of observation. Animals receiving cadmium and zinc together had values similar to those receiving cadmium alone, but zinc tended to reduce the response when given after cessation of cadmium treatment. None of these differences reached the level of statistical significance.

#### 5.1.6. Autoimmune response in kidneys

Whole kidney sections were examined for IgG and C3 deposition by fluorescence microscopy using fluorescein tagged anti-mouse IgG and C3. Six to eight mice from each group were examined at the three observation periods. No evidence of autoimmune reaction involving IgG and C3 deposition was seen in any animal. Fluorescence was noted around the glomerulus and the convoluted tubules, but the amount of fixed antibodies followed the same pattern and appeared to be equal in all treatment groups.

#### 5.1.7. Electron microscopy of kidney

Kidney sections were examined for ultrastructural alterations by transmission electron microscopy. Four animals from each group were examined at 0 and 6 week of observation. Whole nephron and interstitial



TABLE 5.11

## Proliferative Response of Spleen Cells

Treatment	0 week		3 week	
	PHA	Con A	PHA	Con A
None	7.7 $\pm$ 2.9	15.9 $\pm$ 6.6	8.7 $\pm$ 6.2	15.8 $\pm$ 13.7
Cd for 3 wk and none for next 3 wk	12.2 $\pm$ 4.4	26.6 $\pm$ 15.4	17.6 $\pm$ 24.9	27.6 $\pm$ 40.2
Cd with Zn for 3 wk and Zn for next 3 wk	11.1 $\pm$ 8.1	25.9 $\pm$ 26.9	12.0 $\pm$ 9.6	27.4 $\pm$ 27.8
Cd for 3 wk and Zn for next 3 wk			7.2 $\pm$ 3.7	21.1 $\pm$ 21.8
Zn for 3 or 6 wk	9.6 $\pm$ 4.1	17.7 $\pm$ 12.4	9.6 $\pm$ 6.9	18.8 $\pm$ 19.4

Proliferation response is expressed as stimulation index which represents the ratio between mean counts per minute of triplicate cultures stimulated with mitogen divided by the mean counts per minute of triplicate control cultures. The results are expressed as means  $\pm$  SD of 10 observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

tissues were examined. Abnormalities were observed in the proximal tubular epithelial cells in animals treated with cadmium for 3 weeks. As compared to the non-treated animals, the mitochondria were increased in number, swollen and the cisterna distorted. Inside several cells, electron dense bodies were observed (Fig. 5.2). Similar changes were also seen 6 weeks after cessation of cadmium treatment, and in animals receiving cadmium together with zinc. Other areas of kidney, including the glomerulus appeared normal in all groups. Thus cadmium altered the structure of proximal tubular cells which persisted after cessation of treatment and zinc had no effect on this aspect of cadmium toxicity.

#### 5.1.8. Tissue cadmium concentration

Kidney and liver samples from mice killed at 0 and 6 week observation were freeze dried and later analyzed for cadmium content by atomic absorption spectroscopy. Results are shown in Tables 5.12 and 5.13. Kidney and liver cadmium concentration was significantly higher in cadmium treated mice at 0 and 6 week than untreated mice. In cadmium treated mice, kidney cadmium concentration also increased significantly between 0 and 6 weeks, while a trend for decrease in liver cadmium concentration was observed at the same time period. Mice receiving cadmium and zinc together had significantly lower kidney and liver cadmium concentration than animals receiving cadmium alone. Zinc given after cessation of cadmium treatment also tended to decrease cadmium concentration in both the organs.

## ELECTRON MICROSCOPY OF KIDNEY SECTIONS

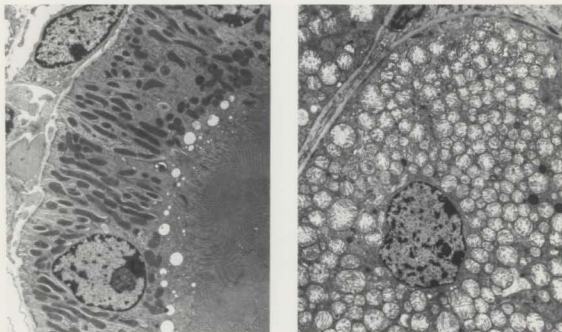


FIG. 5.2

Electron microscopic appearance of proximal tubular cell from a control mouse (left) and cadmium treated (50 ppm cadmium in drinking water for 3 weeks) mouse (right). In the treated animal, mitochondria are increased in number, swollen and the cristernae are distorted. Electron dense materials are present inside the cell. (x 12,000).

TABLE 5.12

Cadmium Concentration in the Kidneys

Treatment	0 week	6 week
	$\mu\text{g/g}$	
None	$0.98 \pm 0.84^a$	$1.41 \pm 0.40^a$
Cd for 3 wk and none for next 6 wk	$8.57 \pm 0.62^b$	$42.15 \pm 3.39^c$
Cd with Zn for 3 wk and Zn for next 6 wk	$5.42 \pm 0.75^d$	$7.29 \pm 1.57^b$
Cd for 3 wk and Zn for next 6 wk		$9.96 \pm 2.11^{bc}$

Values are expressed as mean  $\pm$  SD of eight observations.

In vertical columns values not sharing a common superscript letter are significantly different at  $P < 0.01$  by Duncan's multiple-range test.

In horizontal rows values not sharing a common superscript letter are significantly different at  $P < 0.05$  by Duncan's multiple-range test.

TABLE 5.13

Cadmium Concentration in the Liver

Treatment	0 week	6 week
		$\mu\text{g/g}$
None	$0.61 \pm 0.21^a$	$0.41 \pm 0.14^a$
Cd for 3 wk and none for next 6 wk	$7.30 \pm 0.71^b$	$6.65 \pm 1.18^b$
Cd with Zn for 3 wk and Zn for next 6 wk	$4.57 \pm 0.99^c$	$4.35 \pm 1.9^c$
Cd for 3 wk and Zn for next 6 wk		$5.72 \pm 1.96^{bc}$

Values are expressed as mean  $\pm$  SD of eight observations.

In vertical columns and horizontal rows values not sharing a common superscript letter are significantly different at  $P < 0.05$  by Duncan's multiple-range test.

## 5.2. EXPERIMENT 2

### 5.2.1. General health of mice

As in experiment 1 (Section 5.1.1.), mice were examined at least once a week for external signs of toxicity, weight gain, food disappearance and death. No sign of toxicity was seen in any mice and none died while on treatment. Also on dissection after killing, all organs appeared healthy on gross examination.

Weight of mice was monitored weekly. Results for three time periods are shown in Table 5.14. There were no statistically significant differences between the groups and also mice from all four groups gained weight equally.

Food disappearance was checked for one week immediately prior to killing. Results are shown in Table 5.15. As in the previous experiment (Section 5.1.1. and Table 5.2, page 74), large amounts of food disappeared from the cages and the amount of food pellets disappearing was equal in all groups.

### 5.2.2. Weight and lymphocyte counts in spleen

Spleen was collected at 0 and 3 weeks, weighed and lymphocytes counted on standard Neubauer chamber. Results are shown in Table 5.16. There were no statistically significant differences between the groups. Thus weight and lymphocyte content of spleen was not altered by the cadmium and zinc treatment at the dosage used in this experiment.

TABLE 5.14

Weight of Mice

Treatment	Group	Before treatment	0 week	3 week
gm				
None	I	18.7 $\pm$ 1.3	20.4 $\pm$ 1.2	22.4 $\pm$ 1.7
Cd for 3 wk and none for next 3 wk	II	18.6 $\pm$ 1.5	20.3 $\pm$ 1.3	22.4 $\pm$ 1.1
Cd with Zn for 3 wk and Zn for next 3 wk	III	18.9 $\pm$ 1.0	20.8 $\pm$ 1.2	22.4 $\pm$ 1.3
Zn for 3 or 6 wk	V	19.0 $\pm$ 1.3	20.5 $\pm$ 1.3	22.8 $\pm$ 1.1

Results are expressed as mean  $\pm$  SD of 20 observations before treatment and at 0 week, and 10 at 3 week.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

TABLE 5.15

## Food Disappearance

Treatment	Group	0 week	3 week
gm/day			
None	I	6.4 $\pm$ 1.0	6.8 $\pm$ 1.3
Cd for 3 wk and none for next 3 wk	II	6.3 $\pm$ 0.8	6.4 $\pm$ 1.1
Cd with Zn for 3 wk and Zn for next 3 wk	III	6.0 $\pm$ 1.2	6.4 $\pm$ 1.3
Zn for 3 or 6 wk	V	6.3 $\pm$ 1.1	6.5 $\pm$ 1.1

Food disappearance of individually-caged mice was measured for one week prior to killing. Results are expressed as mean  $\pm$  SD of 20 observations at 0 week, and 10 at 3 week.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.



TABLE 5.16

Weight and Lymphocyte Counts in Spleen

Treatment	Weight		Lymphocyte count	
	0 week	3 week	0 week	3 week
	mg		$\times 10^6$	
None	73.6 $\pm$ 5.0	78.4 $\pm$ 9.5	151.6 $\pm$ 40.2	150.4 $\pm$ 38.8
Cd for 3 wk and none for next 3 wk	76.0 $\pm$ 14.5	75.1 $\pm$ 8.7	162.8 $\pm$ 37.3	153.9 $\pm$ 30.9
Cd with Zn for 3 wk and Zn for next 3 wk	72.4 $\pm$ 7.8	77.6 $\pm$ 13.6	153.4 $\pm$ 43.2	159.4 $\pm$ 46.1
Zn for 3 or 6 wk	73.6 $\pm$ 5.0	71.1 $\pm$ 8.8	163.8 $\pm$ 37.9	149.5 $\pm$ 42.3

Values are expressed as mean  $\pm$  SD of 10 observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

### 5.2.3. Indirect plaque-forming cell response

Indirect plaque-forming cell (PFC) response was assayed immediately after cessation of cadmium treatment (0 week) and 3 weeks later (3 week). Mice were injected with sheep red blood cells (SRBC) 8 days prior to killing and the number of spleen cells producing IgG antibody against the SRBC antigen was counted in a plaque-assay. Results expressed as IgG plaques/ $10^6$  spleen lymphocytes and plaques/spleen are shown in Fig. 5.3 and Table 5.17 respectively. The findings followed the same pattern as for IgM plaque-assay (Fig 5.1, page 84; Table 5.10, page 85). At 0 week of observation, cadmium treated mice had significantly higher IgG PFC number than the other three groups, including that receiving cadmium and zinc together. The number of PFC in the untreated group and those treated with cadmium and zinc together or only zinc was not different from each other. Thus zinc prevented the cadmium-induced increase in IgG PFC response when given along with cadmium. Three weeks after cessation of cadmium treatment (3 week observation), cadmium treated animals still tended to have high IgG PFC number than other three groups and zinc maintained the antagonism, but the differences were statistically not significant.

## 5.3. EXPERIMENT 3

### 5.3.1. General health of mice

As in previous two experiments (Sections 5.1.1. and 5.2.1.) mice were examined at least once a week for external manifestations of toxicity,

## IgG PLAQUES

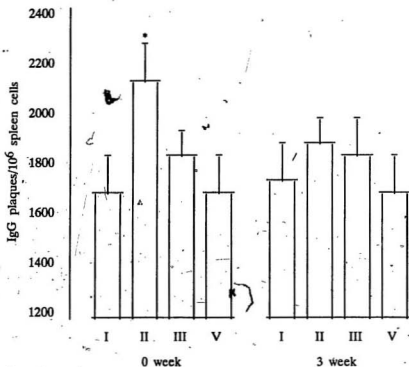


FIG. 5.3

Indirect plaque-forming (IgG) cells response in mice treated with or without zinc. Group I received no treatment; group II, cadmium for 3 weeks and no treatment for next 3 weeks; group III, cadmium with zinc for 3 weeks and zinc for next 3 weeks; and group V, zinc for 6 weeks. Bars represent mean and SD of 10 observations. At 0 week, cadmium treated animals had higher number of plaque-forming cells than the other three groups (\* $P < 0.05$ ). Simultaneous treatment of cadmium and zinc did not result in a greater number of plaque-forming cells. At 3 week, there were no significant differences between the groups.

TABLE 5.17

## Indirect Plaque-forming Cell Response

Treatment	0 week	3 week
	IgG Plaques/spleen	
None	242378 $\pm$ 47632	269728 $\pm$ 66672
Cd for 3 wk and none for next 3 wk	353412 $\pm$ 66444*	293382 $\pm$ 54312
Cd with Zn for 3 wk and Zn for next 3 or 6 wk	279664 $\pm$ 51852	279091 $\pm$ 67875
Zn for 3 or 6 wk	281798 $\pm$ 56483	259596 $\pm$ 75414

Values are expressed as mean  $\pm$  SD of 10 observations.

\*Significantly different at  $P < 0.05$  from other values in the same column by Duncan's multiple-range test.

weight gain, food disappearance and death. No signs of toxicity, both during the treatment and on dissection after killing were seen in any mice.

Weight of the mice at two time periods and food disappearance data for a 7 day period immediately prior to killing are shown in Table 5.18. As in previous two experiments (Table 5.1 page 73, Table 5.2 page 74, Table 5.14 page 93 and Table 5.15 page 94) no statistically significant differences between the groups were observed.

### 5.3.2. Weight and lymphocyte counts in spleen

Immediately after 3 week treatment period, mice were killed, spleens collected, weighed and the lymphocytes counted. Weight of spleen and lymphocyte content for the four groups are shown in Table 5.19. No difference between the treatment groups were observed.

Although in the previous two experiments no differences in spleen weight and lymphocyte content were observed, the procedure was repeated and the data presented as a background, since the other immunological assays were performed on spleen cells.

### 5.3.3. T lymphocyte subsets in spleen

Splenic T lymphocyte subsets were counted by immunofluorescence staining using monoclonal anti-L3T4 and anti-Lyt-2 antibodies. These antibodies identify the murine lymphocyte differentiation antigens CD4 and CD8, which are markers of helper/inducer and suppressor/cytotoxic T cells respectively (Scollay, Bartlett and Shortman, 1984; Lewis and Cahalan

TABLE 5.18

## Weight of Mice and Food Disappearance

Treatment	Group	Before treatment	Weight 0 week	Food disappearance
			gm	gm/day
None	I	19.6 $\pm$ 1.4	23.0 $\pm$ 1.4	6.4 $\pm$ 1.2
Cd for 3 wk	II	19.4 $\pm$ 1.4	23.1 $\pm$ 1.5	6.2 $\pm$ 1.0
Cd with Zn for 3 wk	III	19.6 $\pm$ 1.2	22.9 $\pm$ 1.4	6.5 $\pm$ 1.1
Zn for 3 wk	V	19.7 $\pm$ 1.2	22.9 $\pm$ 1.4	6.3 $\pm$ 1.0

Weight of mice results expressed as mean  $\pm$  SD of eight observations before treatment and at 0 week.

Food disappearance of individually-caged mice was measured for one week prior to killing. Results are expressed as mean  $\pm$  SD of eight observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

TABLE 5.19

Weight and Lymphocyte Count in Spleen

Treatment	Weight	Lymphocyte count
	mg	$\times 10^6$
None	80.7 $\pm$ 19.4	141.3 $\pm$ 25.5
Cd for 3 wk	82.3 $\pm$ 20.3	139.8 $\pm$ 22.5
Cd with Zn for 3 wk	88.6 $\pm$ 12.5	138.3 $\pm$ 22.5
Zn for 3 wk	75.9 $\pm$ 14.1	132.6 $\pm$ 31.7

Values are expressed as mean  $\pm$  SD of eight observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

1988). Results are shown in Table 5.20. Percentage of CD4+ cells was similar in all groups of mice, but that of CD8+ cells was significantly lower in the cadmium treated group than in the other three groups, including that treated with cadmium and zinc together. Concurrent administration of zinc thus prevented the cadmium induced selective reduction of CD8+ cell number in the spleen. The ratio of CD4+ and CD8+ cells, which reflects the balance of immunoregulatory T-lymphocytes, was higher in cadmium treated animals than in all other groups. Zinc prevented this increase.

#### 5.3.4. Suppressor cell activity

The capacity of Con A induced spleen cells to inhibit the mitogenic response of autologous spleen cells to PHA in coculture was measured and the percentage of suppression expressed as the suppressor cell activity. Results are shown in Fig. 5.4. There was a large spread of results and also some values were in the negative range, making interpretation of assay results complex. However, mean suppressor cell activity tended to be lower in mice treated with cadmium than in untreated controls, but the difference was statistically not significant.

### 5.4. EXPERIMENT 4

#### 5.4.1. General health of mice

As in the previous three experiments (Section 5.1.1., 5.2.1 and 5.3.1) mice were examined at least once a week for external signs of toxicity, weight gain, food disappearance and death. All animals were healthy



TABLE 5.20

T-lymphocyte Subsets in the Spleen at 0 week

Treatment	CD4+ cells	CD8+ cells	helper/suppressor
	(L3T4 <sup>+</sup> helper)	(Lyt-2 <sup>+</sup> suppressor)	
		%	
None	34.90 $\pm$ 6.63	24.96 $\pm$ 3.55	1.40 $\pm$ 0.21
Cd for 3 wk	32.35 $\pm$ 5.98	17.24 $\pm$ 2.74*	1.89 $\pm$ 0.32*
Cd with Zn for 3 wk	31.99 $\pm$ 6.00	22.19 $\pm$ 2.79	1.44 $\pm$ 0.19
Zn for 3 wk	33.40 $\pm$ 6.28	23.37 $\pm$ 2.04	1.40 $\pm$ 0.21

Values are expressed as mean  $\pm$  SD of eight observations.

\*Significantly different at  $P < 0.01$  from other values in the same column by Duncan's multiple-range test.

## SUPPRESSOR CELL ACTIVITY

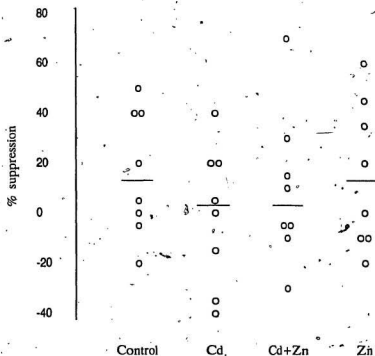


FIG. 54

Suppressor cell activity (% suppression) of Con A stimulated spleen cells. Each point represents one animal and the bars represent the mean value in each group. There were no significant differences ( $P > 0.05$ ) between the groups.

throughout the experimental period and also on dissection after killing no gross pathological lesions were seen in any organs. Weight of mice, at two time periods and food disappearance for a 7 day period immediately prior to killing is shown in Table 5.21. As in previous experiments (Table 5.1 page 73, Table 5.2 page 74, Table 5.14 page 93, Table 5.15 page 94 and Table 5.18 page 100) no statistically significant differences between the groups were observed. However, the food disappearance was considerably lower than before, as mice in this experiment were housed in plastic cages with bedding and as such less food pellets were wasted by the animals.

#### 5.4.2. Weight, lymphocyte and B cell count in spleen

After three week treatment periods, mice were killed, spleens collected, weighed and lymphocytes counted. B cells from spleen cell suspension were stained with FITC-conjugated purified anti-mouse IgG and counted using a fluorescence microscope. Spleen weight, lymphocyte count and B cell count results are shown in Table 5.22. There were no statistically significant differences between the groups, however, total B cell count in the spleen tended to be higher in cadmium treated animals than in the other three groups.

#### 5.4.3. Natural-killer cell activity

Natural-killer cell activity was measured in a 4 and 12 hour chromium release assay using the Moloney virus induced lymphoma cell line YAC-1 labelled with  $^{51}\text{Cr}$  as targets. Results are shown in Fig 5.5. The natural-killer cell activity was lower in cadmium treated animals as compared to

TABLE 5.21

## Weight of Mice and Food Disappearance

Treatment	Group	Weight		Food disappearance
		Before treatment	0 week	
			gm	gm/day
None	I	20.3 $\pm$ 1.5	23.4 $\pm$ 1.5	3.9 $\pm$ 0.6
Cd for 3 wk	II	21.2 $\pm$ 1.3	24.7 $\pm$ 1.6	3.7 $\pm$ 0.4
Cd with Zn for 3 wk	III	20.6 $\pm$ 1.2	24.3 $\pm$ 1.7	3.8 $\pm$ 0.5
Zn for 3 wk	V	20.4 $\pm$ 1.6	24.3 $\pm$ 1.1	3.6 $\pm$ 0.5

Weight of mice results expressed as mean  $\pm$  SD of 10 observations before treatment and at 0 week.

Food disappearance of individually-caged mice were measured for one week prior to killing. Results are expressed as mean  $\pm$  SD of eight observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

TABLE 5.22

Weight, Lymphocyte and B Cell Count in Spleen

Treatment	Weight	Lymphocyte count	B cell	B cell
	mg	X10 <sup>6</sup>	%	X10 <sup>6</sup>
None	67.4 ± 6.8	119.5 ± 46.9	37.7 ± 3.5	44.2 ± 15.2
Cd for 3 wk	72.9 ± 9.1	137.1 ± 49.3	36.8 ± 3.8	50.6 ± 19.3
Cd with Zn for 3 wk	67.4 ± 9.2	121.7 ± 30.2	37.4 ± 3.3	45.5 ± 11.9
Zn for 3 wk	72.2 ± 4.8	123.3 ± 36.7	35.3 ± 4.4	43.4 ± 12.7

The results are expressed as means ± of 10 observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.

## NATURAL-KILLER CELL ACTIVITY

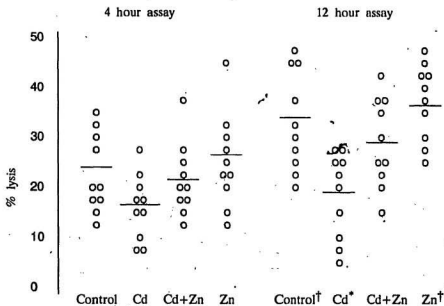


FIG. 5.5

Natural-killer (NK) cell activity (% lysis) in different treatment groups after 4 and 12 hours of incubation of effector lymphocytes with labelled target cells in a ratio of 50:1. Each point represents one animal and the bars represent the mean value in each group. In the 12 hour assay, cadmium treated animals had lower NK cell activity than other three groups (\* $P < 0.05$ ). NK cell activity of groups receiving cadmium with zinc or zinc was not different from control. In 4 hour assay, a similar trend was observed ( $P > 0.05$ ). Between the 4 and 12 hour assay, the mean percentage of target cell lysis increased in the control and zinc treated groups († $P < 0.05$ ), but not in those receiving cadmium alone or with zinc.

untreated controls at both time periods; however it was statistically significant at the 12 hour assay. The activity in groups receiving cadmium with zinc or zinc alone was not different from controls in either time period. Zinc thus prevented the cadmium induced reduction in NK cell activity.

In the culture examined at 12 hours, the mean target cell lysis increased significantly over the 4 hour value in control and zinc treated groups. Cadmium thus prevented the time dependent increase in target cell lysis. Concurrent administration of zinc tended to offset the cadmium induced suppression.

#### 5.4.4. Pokeweed mitogen stimulated IgG production

Pure preparations of lymphocytes were stimulated with the B cell mitogen pokeweed mitogen for 7 days and the level of IgG in the culture supernatant measured by ELISA. Results are shown in Table 5.23. There were no statistically significant differences between the treatment groups.

#### 5.4.5. Liver trace element levels

Liver samples were frozen at  $-20^{\circ}\text{C}$  and later analyzed for multiple trace elements by inductively coupled plasma spectrophotometry. Results are shown in Table 5.24. Liver cadmium concentration was significantly higher in cadmium treated mice as compared to non treated controls. Mice receiving cadmium and zinc together had lower liver cadmium concentrations as compared to those receiving cadmium alone. Zinc level was slightly but significantly elevated in mice treated with cadmium and

TABLE 5.23

Antibody Production by Pokeweed-mitogen Stimulated Lymphocytes

Treatment	IgG
	ng/ml
None	78.7 $\pm$ 14.4
Cd for 3 wk	76.2 $\pm$ 16.4
Cd with Zn for 3 wk	82.0 $\pm$ 15.6
Zn for 3 wk	82.1 $\pm$ 11.3

Values are expressed as mean  $\pm$  SD of 10 observations.

There are no statistically significant differences ( $P > 0.05$ ) between the treatment groups by one-way analysis of variance.



TABLE 5.24

Trace Element Concentrations in Liver at 0 week

Trace elements ( $\mu\text{g/g}$ )	Treatment groups		
	None	cadmium	cadmium + zinc
Cadmium	$0.06 \pm 0.04^a$	$5.64 \pm 1.54^b$	$3.83 \pm 0.63^c$
Zinc	$25.5 \pm 0.8^a$	$27.9 \pm 0.9^b$	$28.3 \pm 1.2^b$
Iron	$63.1 \pm 4.8^a$	$41.4 \pm 3.0^b$	$39.0 \pm 2.5^b$
Copper	$6.41 \pm 0.47^a$	$6.35 \pm 0.32^a$	$6.19 \pm 0.30^a$
Selenium	$3.56 \pm 0.93^a$	$2.36 \pm 1.17^b$	$1.37 \pm 0.61^b$
Aluminum	$2.22 \pm 0.61^a$	$1.10 \pm 0.69^b$	$0.49 \pm 0.37^b$
Manganese	$1.14 \pm 0.08^a$	$1.25 \pm 0.08^b$	$1.23 \pm 0.06^b$
Chromium	$0.16 \pm 0.18^a$	$0.11 \pm 0.03^a$	$0.10 \pm 0.02^a$

Values are expressed as means  $\pm$  SD of six observations.

If horizontal rows values not sharing a common superscripts letter are significantly different at  $P < 0.05$  by Duncan's multiple-range test.

zinc as compared to non treated controls. Concentrations of iron, selenium and aluminum were low in mice treated with cadmium or cadmium with zinc as compared to untreated controls. The manganese level was slightly but significantly elevated in mice receiving cadmium alone or with zinc. Other trace element levels, including copper, were not different in the three treatment groups.

## CHAPTER 6

### DISCUSSION

In this study the effect of a relatively low dose of oral cadmium treatment on the immune system and the interaction between cadmium and zinc on the immune cells were examined in mice. The dose of cadmium used was approximately comparable to possible human exposure. The general health of animals, weight gain and food consumption was not affected by the cadmium and zinc treatment. Also, no abnormalities were seen on gross examination of any organ and no animals died during the experiment. In other studies also where similar doses of cadmium were used (Muller et al., 1979; Koller, Roan and Kerkvliet, 1979; Wesenberg and Wesenberg, 1983; Blakley, 1985; Blakley and Tomar, 1986; Borgman, Au and Chandra, 1986) no general health effects were reported. Malave and DeRuffino (1984) reported a slight reduction in body weight of mice by cadmium treatment at a dose of 50 ppm; however the reduction was more discernible beyond 3 weeks of treatment, the time at which cadmium treatment was stopped in the present experiment. Zinc at the dose of 500 ppm also did not affect the general health of the mice when given alone or together with cadmium. Thus the animals appeared to have tolerated the treatment well.

## 1. EFFECT OF CADMIUM ON IMMUNE RESPONSES

Cadmium at the oral dose of 50 ppm for 3 weeks did not alter the weight of the primary lymphoid organs - the spleen and thymus; nor the lymphocyte counts in the blood, spleen and thymus. Also no effect after 3 and 6 weeks of cessation of cadmium treatment was observed. This finding is in agreement with those of Borgman, Au and Chandra (1986) and Malave and DeRuffino (1984) who also used similar dose of oral cadmium treatment. However, Yamada, Shimizu, Kawamura and Kubota (1981), and Suzuki, Yamada and Shimizu (1981) reported atrophy of the thymus and enlargement of the spleen following cadmium injection. Bozelka, Burkholder and Chang (1978) also reported increase in spleen size following cadmium injection. The difference is probably due to the amount of cadmium delivered to the immune organs depending on the mode of treatment. In contrast to cadmium injection, following oral cadmium treatment a smaller amount of cadmium would be absorbed and delivered to the spleen and thymus. The amount of cadmium was probably less than that required to cause gross structural and morphological alterations in the immune organs.

As a first step to characterize the effect of cadmium on the cellular immune function, IgM and IgG antibody producing cell response to sheep red blood cells, a T cell dependent antigen, was estimated in a plaque assay; and proliferative responses of spleen cells to the T cell mitogens phytohaemagglutinin (PHA) and concanavalin A (Con A) were studied in a 72 hour culture. Assay of antibody production following stimulation with sheep red blood cells is a classical test of immune function in an intact animal. In this test, the functional integrity of the antigen presenting

cells, B cells and the regulatory helper and suppressor T cells are assayed. The direct PFC assays IgM production, while the indirect PFC assay is for IgG production (Jerne et al., 1974; Mishell and Shiigi, 1980). Lymphocyte transformation *in vitro* to mitogens is a useful test to assess cell-mediated immunity. The mitogens PHA and Con A are polyclonal activators of T cells and stimulate the cells to undergo blast transformation without antigen contact. The assay is believed to mimic the *in vivo* immune response (Maluish and Strong, 1986).

In this study, cadmium at a dose of 50 ppm in drinking water for 3 weeks caused a significant increase in IgM and IgG antibody forming cell number. Even 3 weeks after cessation of cadmium treatment the response tended to be higher. Proliferative response of spleen cells to T cell mitogens also tended to be higher in cadmium treated animals, both immediately after 3 weeks of cadmium treatment and 3 weeks after cessation of treatment. These observations are in agreement with those of Malave and DeRuffino (1984) who also used 50 ppm cadmium in drinking water and reported an increase in IgM antibody forming cell response and lymphocyte stimulation response to T cell mitogens. Enhancement of mitogen responsiveness of T cells from animals treated with 50 ppm oral cadmium has also been reported by others (Muller et al., 1979; Blakley, 1985).

In contrast to low dose oral cadmium treatment, cadmium given by injection (Bozelka, Burkholder and Chang, 1978; Fujimaki, 1985), inhalation (Graham et al., 1978; Krzystyniak et al., 1987), or in a large oral dose of 300 ppm for 10 weeks (Koller, Exon and Roan, 1975) reduces the number

of antibody forming cells in the spleen and transformation response of spleen cells to mitogens. Cadmium given by injection causes an immediate and pronounced rise in tissue level, and absorption of cadmium from the lung is more complete than from the gut. Also following parenteral administration, cadmium bypasses the portal circulation and the liver where cadmium is supposedly incorporated to metallothionein and rendered relatively non-toxic before release in the general circulation. Thus in those experiments where cadmium was administered by parenteral routes, a larger amount of cadmium, and probably a more toxic form, was delivered to the immune cells than in the present study. From these studies, it thus appears that a small dose of cadmium increases the number of antibody forming cells and lymphocyte transformation response, whereas a large dose suppresses these responses. Demonstration of augmentation of *in vitro* IgM antibody response by 4 and 8  $\mu\text{M}$  cadmium and its suppression at concentrations of 20 and 40  $\mu\text{M}$  (Fujimaki, Murakami and Kubota, 1982) further supports this concept. Failure to obtain any effect on antibody forming cell number (Muller et al., 1979) or demonstration of reduced number (Blakley, 1985; Borgman, Au and Chandra, 1986) using low doses of cadmium may be due to strain dependent differences, as the expression of heavy metal toxicity is known to vary in different species and strains of laboratory animals (Malave, 1981; Balter, Nieder and Gray, 1982). In the present study and also in the study of Malave and DeRuffino (1984), C57BL/6 mice were used, which is different to the strains used by Muller et al., (1979), Blakley (1985) and Borgman, Au and Chandra (1986).

To further characterize the effects of cadmium on the immune system and to find a possible explanation of the observed increase in IgM and IgG

antibody-forming cells by cadmium, experiments were performed to study the effect of cadmium on the B cell, helper T cell and suppressor T cell numbers and suppressor cell activity. Antibody production by lymphocytes stimulated *in vitro* by the helper T cell dependent B cell mitogen-pokeweed mitogen (PWM) was also assayed. Although antibody is produced by the terminally differentiated B cells - the plasma cells, the process is under the control of an intricate system of regulatory T cells. It is possible that the effect of cadmium on these distinct cell types are dose dependent.

T cells are classically subdivided into two functional types - the helper/inducer and suppressor/cytotoxic T cells, identified by the expression of surface glycoprotein markers CD4 and CD8 respectively. In the murine system CD4<sup>+</sup> and CD8<sup>+</sup> T cells can be identified by the monoclonal antibodies against L3T4 and Lyt-2 antigens respectively (Adkin et al., 1987; Lewis and Cahalan, 1988). Expression of L3T4 and Lyt-2 antigens by the murine T cells is mutually exclusive both with respect to functional T cell clones and with respect to splenic T cells (Dialynas, Wilde, Marrack, Pierres, Wall, Havran, Otten, Loken, Pierres, Kappeler and Fitch, 1983). In this study, it was found that 50 ppm cadmium treatment for 3 weeks selectively reduced the number of CD8<sup>+</sup> T cells in the spleen. CD8<sup>+</sup> cells are known to include two distinct functional cell types, the suppressor T cells which are involved in immunoregulation and the cytotoxic T cells which are the effectors of T cell mediated antigen-specific cytotoxicity. Reduction in CD8<sup>+</sup> cells may be due to reduction of either or both cell types, however, it is more likely that the suppressor T cells were affected by cadmium as in the cadmium treated animals the

suppressor cell activity tended to be lower. Malave and DeRuffino (1984) also reported reduction of suppressor cell activity by oral 50 ppm cadmium treatment in mice. The effect of cadmium on suppressor T cells was possibly a direct selective effect of cadmium on this subset and not an indirect effect on the entire lymphoid system due to stress with increased secretion of corticosteroids. The number of suppressor T cells was only reduced in cadmium treated mice and not in those receiving cadmium with zinc.

B cell function may also be influenced by cadmium. Fujimaki (1985) reported direct inactivation of B cells resulting in reduction of IgM antibody response following a single large dose cadmium injection. At lower parenteral doses, cadmium is reported to cause slight splenomegaly and increase in B cell numbers in the spleen (Bozelka, Burkholder and Chang, 1978); while at oral doses ranging from 50 to 300 ppm in drinking water cadmium increases lymphocyte proliferation response to B cell mitogens (Muller et al., 1979; Koller, Roan and Kerkvliet, 1979; Blakley, 1985), follicular hyperplasia of lymphoid tissues with marked B cell proliferation associated with antibody excess (Powell, Joshi, Dwivedi and Green, 1979) and deposition of IgG in renal glomeruli causing diffuse membranous nephropathy (Joshi et al., 1981). It has been postulated on the basis of the latter findings that cadmium may act as a polyclonal B cell stimulant or act synergistically with other B cell stimulants. However, in the present study, the number of B cells in the spleen was not altered by cadmium treatment. Also on stimulation with PWM, lymphocytes from cadmium treated mice produced antibodies in amounts comparable to those observed in the untreated mice. Thus at the dose of 50 ppm for 3 weeks,



cadmium did not alter the B cell number or functional activity of the B cell itself. So it may be derived that the apparent increase in immune response observed following cadmium treatment was probably due to a selective reduction of cells carrying CD8 surface antigen which identifies a subset of cells with suppressor activity, and not due to any direct effect of cadmium on the B cells.

On the basis of findings of this study and those of others, and taking into consideration that cadmium is a cellular toxic element, it can be proposed that cadmium has important effects on immunoregulatory T cell number and function; at a relatively low dose cadmium affects the  $CD8^+$  T cells with suppressor function and, thereby augments measurable immune functions like antibody production against heterologous antigens and lymphocyte transformation response to mitogens, whereas at a high dose all cell types are affected, resulting in general impairment of immune responses. This model explains most of the experimental findings on cadmium immunotoxicity and resolves the ambiguity of the findings reported by different investigators.

The effect of cadmium on natural-killer cell activity was also studied. Natural-killer cells are an important effector mechanism of non-specific immunity. They can recognize surface changes in virus-infected or malignant cells and lyse them. Natural-killer cells do not require prior sensitization to become cytotoxic and their activity is not restricted by major histocompatibility complex (MHC) antigens. Normally the immune cells recognize antigens only when presented in conjunction with an MHC molecule; for example the  $CD4^+$  (helper/inducer) T cells recognize foreign

antigens when presented with self MHC class II molecule, while CD8<sup>+</sup> (suppressor/cytotoxic) T cells recognize antigens presented in conjunction with class I molecules. In contrast, the natural-killer cells do not require either class I or II molecules for recognition of antigens. Thus *in vivo* they function as an effective surveillance mechanism against infection, particularly viral, and emergence of tumour cells. Natural-killer cells lyse the target cells by a complex mechanism. They first attach to the target by a receptor, then lyse the cell in a Ca<sup>++</sup>-dependent mechanism by orienting the granules, Golgi apparatus and microtubular organizing complex toward the target and releasing the lethal pore-forming protein perforin and other cytotoxic factors. The killer cells themselves survive the encounter with target cell and go on to kill again (Marx, 1986; Young and Cohn, 1988).

In this study it was demonstrated that cadmium at the oral dose of 50 ppm for 3 weeks reduces the natural-killer cell activity in mice. At 12 hours, the reduction was statistically significant, but a similar trend was observed also at 4 hours. It is possible that the low natural-killer cell activity was due to a reduction in the total number of natural-killer cells by cadmium. However, it seems that more than a simple numerical reduction of natural-killer cells occurred, as cadmium treated animals not only had a low natural-killer activity but also failed to show an increase in activity with time. In contrast to other treatment groups, the percentage of target cell lysis was essentially the same in cadmium treated animals at 4 and 12 hours. It is known that one natural-killer cell can lyse many targets by lysing them successively one after another. Thus in *in vitro* culture the percentage of target cell lysis increases with time

(Kiessling, Klein and Wigzell, 1975). The results of this study show that cadmium treatment prevented such recruitment and as a whole reduced the efficiency of the killing process.

## 6.2. POSSIBLE MECHANISMS OF CADMIUM IMMUNOTOXICITY

In this study it was demonstrated that a relatively low dose of cadmium selectively affects the suppressor T cells and natural-killer cells resulting in increased IgM and IgG plaque-forming cell number and reduced natural-killer cell activity. The cause or mechanism of the selective effects of cadmium on these two cell types were not investigated, but several mechanisms may be postulated to explain the observation.

It is known that lymphocytes cultured *in vitro* take up a larger amount of cadmium as compared to other formed elements of blood (Hildebrand and Cram, 1979). But in leukocytes the inducible intracellular metal binding protein metallothionein is probably not induced to the extent as seen in other tissues like the kidneys and liver (Bannerjee, Onosaka and Cherian, 1982). Metallothionein is known to bind free intracellular cadmium with high affinity and keeps it in a non-ionic form which is non-toxic. It is possible that the suppressor T cells and the natural-killer cells either take up larger amounts of cadmium compared to other types of lymphocytes or the metallothionein gene in these cells is not induced to the same extent as in other cells. As a result, large amounts of the toxic ionic form of cadmium may accumulate inside these cells resulting in their loss of functional competence. Alternatively, the subcellular machinery

unique to suppressor and natural-killer cells may be more susceptible to cadmium toxicity than others.

At the subcellular level, cadmium may interfere with the function of enzymes and structural proteins. Cadmium may displace zinc from certain enzymes essential for cellular function and render them inactive. DNA polymerase, RNA polymerase and nucleoside phosphorylase are some of the zinc-dependent enzymes that are crucial catalysts involved in the replication and transcription of DNA during cell division, and thus are important at various stages of immune responses (Lieberman et al., 1963; Ballester and Prasad, 1983). Due to mutual antagonism, displacement of zinc by cadmium from the catalytic part of these enzymes is possible.

Zinc is known to be essential for the structural integrity of nucleic acids (Hambidge, Casey and Krebs, 1986), some regulatory proteins involved in gene expression (Klug and Rhodes, 1987), plasma membranes and cytoskeletal elements - microtubules and microfilaments (Chvapil, 1976). Nucleic acids and the regulatory proteins are of obvious importance in all aspects of cellular functions including those of the immune cells. Microtubules and microfilaments are essential for many cell membrane associated phenomena including antigen processing and multiplication. They are also required for movement of the cells and their organelles. It is possible that due to the physical and chemical similarity between cadmium and zinc, cadmium may displace zinc from these sites and affect their structural integrity and functions.

Large amounts of intracellular cadmium may also cause a relative zinc deficiency in the cells. Cadmium is known to be a strong inducer of

metallothionein synthesis, and metallothionein is known to bind various heavy metals particularly cadmium, zinc and copper with high affinity (Hamer, 1986; Dunn, Blalock and Cousins, 1987). Normally, the small amount of metallothionein that is present inside the cells is thought to regulate the movement of zinc and copper to active sites, like enzymes, where they are required. Nature has devised such a mechanism as zinc and copper ions are toxic. It is possible that following cadmium entry into the lymphocytes, the newly formed metallothionein binds zinc in addition to cadmium and thus sequesters it from sites where it is required.

Cadmium may inhibit the synthesis of interleukins, which are involved in immune responses. Reduction of interleukin synthesis is possible as cadmium is known to adversely affect enzyme functions and protein synthesis in various tissues (Kostial, 1986), and cadmium induced reduction in synthesis of one lymphokine - macrophage migration inhibitory factor has been reported (Kiremidjian-Schumacher et al., 1981).

Cadmium may also interfere with calcium metabolism in the affected lymphocytes. Many cellular functions including those involved in immune response are triggered by transmembrane movement of calcium. For the natural-killer cells, target cell lysis is known to be associated with a massive increase in calcium in the killer cell (Young and Cohn, 1988). Cadmium is known to inhibit calcium uptake by epithelial cells (Verboost, Flik, Lotk and Bonga, 1987). A similar effect may occur in the lymphocytes with reduction in the intracellular pool of available calcium.

### 6.3. EFFECT OF ZINC ON CADMIUM-INDUCED IMMUNOPATHOLOGY

Cadmium and zinc have many physical and chemical similarities. Both the metals are found together in nature and also in biological tissues where they are thought to compete for common ligands. Many toxic effects of cadmium have been related to altered zinc metabolism and zinc is often considered to be a metabolic antagonist of cadmium. In this study, one major objective was to characterize the effects of a relatively large dose of zinc on cadmium-induced immunopathology. Thus in some animal groups, 500 ppm zinc was given in drinking water together with or following cadmium treatment.

The results demonstrate that zinc prevents the cadmium-induced alterations in immune functions. It was shown that enhancement of IgM and IgG antibody forming cell number induced by cadmium was prevented by concurrent treatment of zinc. In animals given zinc after cadmium administration, the lymphocyte proliferation response to T-cell mitogens also tended to be lower than in other treatment groups. These findings are in agreement with those of Malave and DeRuffino (1984) who also demonstrated prevention of cadmium-induced increase in IgM antibody response by zinc injection. However, Shippee et al., (1983) using large doses of cadmium and zinc by injection in mice failed to show any protective effect of zinc on cadmium-induced alteration of immune responses. It is likely that in acute toxicity studies in which large parenteral doses of cadmium are used, the amount delivered to the immune cells is very large which results in irreversible alterations of immune cells. But in small oral doses, the immune functions are altered for a short time

period and recover after cessation of cadmium treatment. Nevertheless, in the same study Shippee et al., (1983) found that zinc completely prevented the cadmium induced mortality when given together with cadmium.

It was also demonstrated in the present study that the antagonism between cadmium and zinc occurred at the level of  $CD8^+$  T cells. Whereas animals receiving only cadmium had a significant reduction of  $CD8^+$  T cell number, those receiving cadmium and zinc together had  $CD8^+$  T cell counts comparable to the animals receiving no treatment. Also, the  $CD4^+/CD8^+$  ratio, which denotes the balance between the immunoregulatory helper and suppressor T cells, was not altered in animals receiving cadmium with zinc, while the ratio was increased by cadmium treatment alone. This effect was probably the result of a specific interaction between cadmium and zinc, as zinc treatment alone did not cause any alterations of T cell subset number. The B cell count and antibody production by lymphocytes on PWM stimulation was not altered by zinc treatment alone or with cadmium.

Suppression of natural-killer activity by cadmium was also prevented by concurrent administration of zinc and reached statistical significance in the 12 hour assay. In the 4 hour assay, although the suppression of natural-killer cell activity by cadmium was not significant, nevertheless zinc therapy did create some antagonism. Also the increase in mean target cell lysis between 4 and 12 hour assay tended to be higher in animals receiving cadmium with zinc as compared to those receiving only cadmium. The prevention of cadmium induced suppression of natural-killer cell activity by zinc reflects a specific interaction between the two elements

and not a non-specific stimulation of natural-killer cell activity by zinc, as zinc treatment alone did not increase the natural-killer cell activity.

#### 6.4. POSSIBLE MECHANISMS OF CADMIUM-ZINC INTERACTION

Various mechanisms may be postulated to explain the interaction between cadmium and zinc on the immune cells. It is possible that the selective effect of cadmium on suppressor T cells and natural-killer cells was due to uptake of cadmium by these cells in amounts larger than other cell types of the immune system. If that is the case, then large amounts of zinc may have antagonized this selective cadmium accumulation. Such an occurrence is likely as *in vitro* studies have shown that the uptake and toxicity of cadmium to cultured cell lines are reduced by large doses of zinc (Meshitsuka, Ishizawa and Nose, 1987).

At the subcellular level also cadmium and zinc may antagonize each other at various sites and steps. It is possible that cadmium displaces zinc from certain enzymes or cellular structural components crucial to immune functions and a large dose of zinc prevents or reverses the phenomenon. Pretreatment with zinc is known to prevent cadmium-induced alterations in some enzyme activities (Watanabe et al., 1985; Suzuki and Cherian, 1987), and both cadmium and zinc have a strong affinity for the cytoskeletal elements (Chvapil, 1976). It is possible that the cytotoxic effect of cadmium was due to a conditional zinc deficiency inside the cells and the large dose of zinc abrogated this effect.

Zinc can also interfere with cadmium metabolism. Zinc treatment is known to alter the shift of cadmium from one body compartment to



another and also slow other cadmium distribution processes (Marcus, 1982; Jones, Jones, Holscher and Vaughn, 1988). Alteration of cadmium metabolism by zinc is probably mediated through metallothionein as both the metals are known to have common binding sites in the protein. It is possible that zinc treatment induced synthesis of a type of metallothionein that bound cadmium with high affinity and prevented cadmium toxicity to essential structural and functional components of the cell.

Zinc-cadmium interaction through effects on calcium metabolism is also possible; zinc may prevent the cadmium induced reduction of calcium transport across cell membranes.

#### 6.5. EFFECT OF CADMIUM AND ZINC ON KIDNEYS

Renal dysfunction characterized by low molecular weight proteinuria is long regarded as the characteristic feature of cadmium toxicity. Several studies have shown that the proximal tubular cells and possibly the glomeruli are involved in chronic cadmium toxicity, both in man and laboratory animals (Friberg, 1984; Piscator, 1986). Although the precise mechanism of cadmium nephrotoxicity is not known, the immune system has often been implicated (Joshi et al., 1981; Bernard et al., 1984). In the present study, kidney sections were examined for deposition of IgG and complement C3. It was hypothesized that cadmium containing complexes deposited either in the glomeruli or in the renal tubules may result in an autoimmune response. No evidence of any autoimmune reaction involving IgG and complement C3 deposition was seen in the kidneys at any

observation time. It thus appears that nephropathy induced by low doses of cadmium is not mediated by the immunologic mechanisms.

On electron microscopic examination, the mitochondria of the proximal tubular cells from cadmium treated mice were found to be increased in number, swollen and the cisternae were distorted. Inside several cells, electron dense materials were observed. Although the electron dense bodies were not further characterized, they probably represent aggregated cadmium-metallothionein complexes. Similar findings were also reported by others (Fowler, 1983; Borgman, Au and Chandra, 1987). The involvement of the proximal tubular cells can be explained from the known kinetics of cadmium in the body. Following gastrointestinal absorption, cadmium is incorporated with metallothionein in the liver and then released slowly into the circulation (Kostial, 1986). Cadmium-metallothionein complex in the blood is probably filtered through the glomeruli and reabsorbed from the proximal tubules (Piscator, 1986). Microinjection studies in rat nephrons show that cadmium compounds are preferentially taken up by the proximal tubular epithelial cells (Felley-Bosco and Diezi, 1987). Inside the tubules, cadmium is released from the metallothionein and incorporated again into newly synthesized metallothionein. During the process of degradation of reabsorbed cadmium-metallothionein complex and incorporation into newly synthesized metallothionein, a toxic ionic form of cadmium may appear transiently inside the tubular epithelial cells resulting in their damage. Increased number of mitochondria seen inside the cells may also reflect the heightened metabolic activity of these cells loaded with additional cadmium.

## 6.6. TRACE ELEMENT LEVELS IN LIVER AND KIDNEYS

The kidney and liver cadmium concentrations were elevated in cadmium treated animals. The kidney cadmium concentration was comparable to the observation reported by others where a similar dose and route of treatment was used (Koller, Exon and Roan, 1976; Blakley, 1985; Borgman, Au and Chandra, 1986). Immediately after cessation of cadmium treatment, cadmium concentration in the liver and kidneys were almost equivalent, whereas 6 weeks after cessation of cadmium treatment the concentration in kidneys were almost double to that in liver. Similar changes in liver and kidney cadmium distribution following cessation of treatment have also been reported by Borgman, Au and Chandra (1986). From these results, it appears that after cessation of treatment, cadmium is gradually lost from the liver and taken up by the kidneys. This is in agreement with the prevailing concept of cadmium kinetics in the body (Friberg, 1984; Piscator, 1986). In the kidneys, cadmium is probably deposited in the proximal tubular epithelial cells bound to metallothionein. Cadmium being a strong inducer of metallothionein synthesis, will increase its level further and allow more cadmium to be deposited. The slight increase in kidney weight seen after cessation of cadmium treatment probably was caused by increased cadmium load and concomitant increase in metallothionein level.

Zinc given together with cadmium resulted in lower liver and kidney cadmium concentration as compared to cadmium alone. Even zinc treatment after cessation of cadmium treatment resulted in lowering of tissue cadmium levels. Similar observation in ruminants has been reported by others (Lamphere, Dorn, Reddy and Meyer, 1984; Reddy, Mohammad,

Ganjam, Martino and Brown, 1987). It is possible that zinc altered the distribution of cadmium in the body. This is likely as zinc is known to shift cadmium from one body compartment to the other (Marcus, 1982). Alteration of cadmium absorption and thus its total body burden by zinc is unlikely as the results of kinetic studies on cadmium absorption argue against competition between cadmium and zinc at the level of uptake from the intestinal lumen (Foulkes, 1985).

Among changes in other trace elements, the most striking was the reduction of liver iron concentration in cadmium treated mice. Friel, Borgman and Chandra (1987) also reported similar findings, however, unlike their finding, copper level was not seen to be decreased by cadmium. Reduction of iron by cadmium treatment is possible as cadmium may interfere with iron absorption or increase loss of iron in the urine (Weigel, Elmadfa and Jager, 1984). Manganese level was slightly elevated; and selenium level was decreased following cadmium treatment. Schroeder and Nason (1974) also reported an increase in liver manganese following cadmium treatment in rats. Zinc treatment did not alter any of the trace element levels including copper.

#### 6.7. HEALTH IMPLICATION OF THE OBSERVATIONS

The implications of the decrease in the number and function of suppressor T cells and natural-killer cell activity in mice by a relatively low dose of oral cadmium and its prevention by zinc are manifold.

Suppressor T cells are known to downregulate the immune response following an antigen challenge and thus protect against the development of

an inappropriate excessive immune response. Loss of suppressor T cell function in man is known to predispose to conditions like atopic diseases and autoimmunity. In this study it was demonstrated that a relatively low dose of cadmium selectively affects suppressor T cells and thus increases the ratio of helper to suppressor T cells. This finding is more relevant to possible human cadmium exposure, as in man low levels of exposure are likely to occur. Although it is not appropriate to infer from this animal study that immunological effects of cadmium do occur in man at the present levels of exposure among the general population, it is interesting to note that atopic diseases are more prevalent among smokers (Zetterstrom, Osterman, Machado and Johansson, 1981; Burrows, Halonen, Lebowitz, Knudson and Barbee, 1982) who are known to be exposed to large amounts of cadmium, and also among people living in industrialized countries (Krause, 1986) where cadmium exposure is more likely to occur. Human atopic disorders are predisposed by various genetic and environmental influences (Bjorksten, 1987). Suppressor T cells also play an important role in this group of diseases and studies have shown that loss of suppressor T cell number and function precedes and predisposes to the development of atopic diseases (Chandra and Baker, 1983; Tainio, 1985). Susceptibility of suppressor T cells to cadmium toxicity thus raises the possibility that this metal may be an important environmental influence on the development of human atopic disorders. Cadmium immunotoxicity may also be involved in other diseases like infection and malignancy. Although the health effects of cadmium are not greatly appreciated at present, its long half-life makes it possible that chronic exposure to low, and presumably, non-toxic doses of the metal may, in fact, lead to

accumulation of levels that can have adverse effects on the immune system of man.

Loss of natural-killer cell function by cadmium may contribute to the development of infection and malignancy. Observation of increased incidence of infections, including viral, in cadmium treated animals may be contributed to by loss of natural-killer cell activity in addition to other immune cell dysfunctions. Cadmium treated animals are also known to have increased malignancies. In rats and mice, cadmium given by injection, inhalation or in large oral doses produces malignancy at various sites (Haddow et al., 1964; Bomhard, Vogel and Loser, 1987). Prolonged inhalation of cadmium chloride fumes at exposure levels within the current occupational limits has been demonstrated to cause a dose dependent increase in lung cancer in rats (Takenaka et al., 1983). In humans the carcinogenic potential of cadmium is controversial, but a recent report in which a large cohort was studied showed a definite increase in lung cancer in industrial workers exposed to cadmium (Thun et al., 1985). Although these studies show that cadmium may cause cancer, the pathogenesis of carcinogenicity has not yet been clarified. It is possible that the loss of natural-killer cell function, combined with impairment of other aspects of immune responsiveness contributes to the development and persistence of cancer in experimental animals and probably in man exposed to cadmium for a long period. The results of this study thus provide a plausible explanation of this important aspect of cadmium toxicity.

The interaction between cadmium and zinc on the immune cells was the other significant finding of this study. The toxic effect of cadmium is

known to be related in part to zinc deficiencies and various studies have previously demonstrated that large doses of zinc can prevent some acute and chronic manifestations of cadmium toxicity (Parizek, 1957; Gunn, Gould and Anderson, 1963b; Perry, Erlanger and Perry, 1977; Ahokas, Dilts and LaHaye, 1980; Shippee et al., 1983; Warner et al., 1984; Marlow and Freeman, 1987). This study shows that even cellular effects of cadmium can be prevented by a moderately large dose of zinc. Although animal studies cannot be extrapolated directly to humans, a similar interaction may also occur in man. Thus it raises the possibility that zinc may have potential therapeutic benefit in situations of cadmium toxicity. Thus it can be proposed that zinc be considered as a therapeutic agent for prevention and treatment of clinical manifestations due to acute or chronic cadmium exposure.

On the other hand, deficiency of zinc may also increase the toxicity of cadmium. Although direct experimental evidence supporting this concept is not available, the close relationship between cadmium and zinc makes such a possibility likely. Recent reports show that in large segments of the population, particularly among the independently-living elderly in developed countries, zinc nutriture is less than adequate (Sandstead, Henriksen, Greger, Prasad and Good, 1982). This hitherto unrecognized zinc deficiency has been shown to be associated with reduced immunocompetence (Bogden, Oleske, Munves, Lavenhar, Bruening, Kemp, Holding, Denny and Loria, 1987) and is thought to contribute to the development of some diseases like cancer and autoimmunity which are more prevalent in old age (Chandra, 1985). It is possible that inadvertent cadmium exposure through

food or cigarette smoking in this segment of the population may aggravate the immunological effects of zinc deficiency.

#### 6.8. EFFECT OF ZINC THERAPY ON IMMUNITY AMONG SMOKERS

As an extension of the present work, the effect of zinc therapy on immunity among cigarette smokers was studied. Smokers were chosen as the study population, as cigarettes contain a large amount of cadmium and smokers are known to have alterations of immune functions. One cigarette generally contains about 2  $\mu\text{g}$  of cadmium, of which 5 to 10 percent is inhaled (Perry, Thind and Perry, 1976). The cadmium present in cigarettes is dispersed into fine particles along with the smoke and reaches the lower respiratory tract from where it is more completely absorbed. In contrast to only 3 to 8 per cent cadmium absorption from the gut, absorption from the lung is usually about 50 per cent (Elinder et al., 1976). Assuming this 50 percent absorption, one pack of 20 cigarettes will thus contribute 1 to 2  $\mu\text{g}$  cadmium, and one pack year (pack years = number of packets smoked daily x number of years smoked) of smoking will increase the body cadmium burden by about 0.3 to 0.7 mg. Considering the total body burden of cadmium, which is 15 to 30 mg, this is a very large amount. Also cadmium absorbed from the lung reaches the general circulation directly, bypassing the liver where it is partially detoxified by incorporation into metallothionein.

The immune dysfunctions described in heavy smokers include increase in leukocyte and lymphocyte counts (Hughes, Haslam, Townsend, Turner-Warwick, 1985; Petitti and Kipp, 1986); reduced immunoglobulin levels



(Gerrard, Heiner, Ko, Mink, Meyers and Dosman, 1980); reduced T cell and helper T cell number, and increased suppressor T cell number (Ginns, Goldenheim, Miller, Burton, Gillick, Colvin, Goldstein, Kung, Hurwitz and Kazemi, 1982); increased lymphocyte transformation response to T cell mitogens (Silverman, Potvin, Alexander and Chretien, 1975); and reduced natural-killer cell activity (Phillips, Marshall, Brown and Thompson, 1985). These immune dysfunctions, particularly suppressed natural-killer cell activity and altered immunoregulatory T cell profile could predispose cigarette smokers to chronic lung infections and malignancies (Ginns et al., 1982; Phillips et al., 1985).

While the effect of smoking on the immune system function and its causal association with various disease processes are well established, the chemical compound(s) in cigarette which causes these immune alterations is not precisely identified. Among the numerous constituents, cadmium is likely to cause the immunosuppression, as cadmium is known to have a strong affinity for human lymphocytes (Hildebrand and Cram, 1979), cigarette smoke contains a large amount of cadmium, and in the present mouse study it has been demonstrated that cadmium at low doses also affects the immune system. Since the cadmium induced immunopathology in mice was shown to be prevented by large doses of zinc, so it was logical to study the effect of zinc supplementation on immunity among cigarette smokers.

Fourteen healthy smokers and 10 non-smokers, aged between 23 and 35 years, participated in the study. They were all free from any overt diseases and none were taking any dietary supplementation or medications

known to alter immune functions. The smokers were consuming at least one packet of cigarettes-a-day for a minimum of 6 years. Due to their young age, however, the pack-years of smoking were not large and varied from 6 to 20. Young smokers only were studied to exclude the natural age-related decline of immunocompetence. The smokers and non-smokers were matched for age, sex and body build (Table 6.1). The subjects were given 50 mg elemental zinc twice-a-day for 4 weeks, and blood was collected immediately before and after zinc supplementation for study of immune functions, lipid profile and trace element levels. The subjects were asked not to make any noticeable changes in their smoking habit, diet or physical activity during the study period. The study protocol was approved by the Human Investigation Committee of the Faculty of Medicine, Memorial University of Newfoundland.

The laboratory methods followed were essentially identical to the mouse study described in Chapter 4, with some modifications as described below. Blood leukocytes and lymphocytes were counted using standard Neubauer counting chamber (Section 4.3.1). The lymphocytes were separated by density gradient centrifugation (Section 4.8.1), and the number of T cells, helper/inducer T cells, suppressor/cytotoxic T cells, natural-killer cells and B cells were counted by direct immunofluorescence microscopy (Section 4.7.1) using appropriate specific monoclonal or polyclonal antibodies. The monoclonal antibodies used were phycoerythrin (PE)-conjugated anti-Leu-4 for T cells (CD3), fluorescein isothiocyanate (FITC)-conjugated anti-Leu-3a for helper/inducer T cells (CD4), PE-conjugated anti-Leu-2a for suppressor/cytotoxic T cells (CD8) and PE-conjugated anti-Leu-11c for natural-killer cells (CD16) (all from Becton Dickinson

TABLE 6.1  
Characteristics of the Study Population

	Non-smokers	Smokers
Number of subjects	10	14
Sex (male : female)	4 : 6	3 : 11
Age (yrs), mean (range)	28.7 (23-35)	27.2 (23-34)
Height (cm), mean $\pm$ sd	165.6 $\pm$ 10.4	163.6 $\pm$ 12.6
Weight (Kg), mean $\pm$ sd	60.3 $\pm$ 12.5	61.3 $\pm$ 10.6
Mid-arm circumference (cm), mean $\pm$ sd	26.2 $\pm$ 3.4	27.0 $\pm$ 4.2
Triceps skinfold thickness (mm), mean $\pm$ sd	17.0 $\pm$ 3.5	18.5 $\pm$ 6.4
Cigarettes smoked per day mean (range)	-	23.2 (20-30)
Pack-years of smoking mean (range)	-	13.6 (6-20)

Immunocytometry System, Mountain View, California). For B cells, FITC-conjugated goat anti-human immunoglobulins (IgG, IgM, IgA) F(ab')<sub>2</sub> (Ortho Diagnostic System, Raritan, New Jersey) was used. Lymphocyte transformation response of peripheral blood lymphocytes to the T cell mitogens PHA and Con A were assayed in a 72 hour culture (Section 4.5.2); and natural-killer cell activity was assayed in a 4-hour chromium release assay (Section 4.8) using the human leukemia cell line K562 as targets. Plasma samples were analyzed for trace element levels by spectrophotometry (4.12) and serum lipid levels were estimated by standard biochemical and electrophoretic methods. Data were analyzed on the Memorial University VAX/VMS mainframe computer using the statistical Packages MINITAB (Ryan, Joiner and Ryan 1985a; Ryan, Joiner and Ryan 1985b) and SAS (Cody and Smith, 1985). The grouped t-test was used to compare the smokers and non-smokers values, while paired t-test was used to compare the pre- and post-zinc supplementation values. Significance level used was 0.05.

Results of the study are shown in Tables 6.2, 6.3 and 6.4, and Figures 6.1, 6.2 and 6.3. In smokers the leukocyte, lymphocyte, T cell, helper/inducer T cell and natural killer count was unaltered; while the suppressor/cytotoxic T cell count was high. The results are in agreement with those reported by others (Hughes et al., 1985; Petitti and Kipp, 1986; Ginns et al., 1982). Zinc treatment reduced the suppressor/cytotoxic T cell count of smokers to values seen in non-smokers and increased the ratio of helper to suppressor T cells. There is a discordance between the results of CD8<sup>+</sup> suppressor/cytotoxic T cell number in the animal and human experiment. The reason for this difference is not clear. Future work will

TABLE 6.2  
Circulating Lymphocyte Profile

	Non-smokers (n=10)		Smokers (n=14)	
	Before Zn	After Zn	Before Zn	After Zn
Leukocyte $\times 10^9/L$	6.9 $\pm$ 1.1	7.5 $\pm$ 1.7	7.3 $\pm$ 1.3	7.8 $\pm$ 2.2
Lymphocyte $\times 10^9/L$	2.4 $\pm$ 0.6	2.4 $\pm$ 0.6	2.9 $\pm$ 0.6 <sup>§</sup>	2.7 $\pm$ 0.8
T cell %	65.0 $\pm$ 8.3	67.9 $\pm$ 5.0	65.1 $\pm$ 8.5	66.4 $\pm$ 4.8
T helper %	43.4 $\pm$ 7.4	41.2 $\pm$ 8.7	45.1 $\pm$ 10.3	46.9 $\pm$ 9.5
T suppressor %	24.7 $\pm$ 4.0	24.6 $\pm$ 2.9	29.4 $\pm$ 6.1*	25.2 $\pm$ 3.5
help/supp ratio	1.8 $\pm$ 0.3	1.7 $\pm$ 0.5	1.6 $\pm$ 0.4	1.9 $\pm$ 0.4 <sup>†</sup>
B cell %	32.3 $\pm$ 5.9	30.1 $\pm$ 4.1	27.2 $\pm$ 5.7*	25.3 $\pm$ 3.5
NK cell, %	13.1 $\pm$ 2.9	13.7 $\pm$ 2.0	11.7 $\pm$ 3.2	12.9 $\pm$ 3.9

Results are expressed as mean  $\pm$  sd.

\*P<0.05 vs non-smokers by grouped t-test.

†P<0.05 vs pretreatment value by paired t-test.

§P=0.05 vs non-smokers by grouped t-test.

TABLE 6.3  
Serum Lipid Profile

	Non-smokers (n=10)		Smokers (n=14)	
	Before Zn	After Zn	Before Zn	After Zn
Total-Chl (mmol/L)	5.1 ± 0.9	4.8 ± 0.9	5.3 ± 1.1	5.1 ± 1.0
LDL-Chl (mmol/L)	3.0 ± 0.8	2.7 ± 0.7	3.4 ± 1.1	3.1 ± 1.0 <sup>†</sup>
HDL-Chl (mmol/L)	1.5 ± 0.5	1.5 ± 0.4	1.3 ± 0.3	1.3 ± 0.3
Triglyceride	1.3 ± 0.9	1.4 ± 1.0	1.1 ± 0.4	1.4 ± 0.8
Risk factor	3.7 ± 1.0	3.4 ± 0.6	4.3 ± 1.7	4.1 ± 1.6

Results are expressed as mean ± sd.

<sup>†</sup>P<0.05 vs pretreatment value by paired t-test.

TABLE 6.4  
Trace Element Levels in Plasma

	Non-smokers (n=10)		Smokers (n=14)	
	Before Zn	After Zn	Before Zn	After Zn
	$\mu\text{g/dl}$			
Zinc	88.3 $\pm$ 13.8	130.9 $\pm$ 33.8 <sup>†</sup>	85.9 $\pm$ 10.5	101.5 $\pm$ 17.8 <sup>†</sup>
Copper	99.3 $\pm$ 23.3	92.7 $\pm$ 18.7 <sup>†</sup>	116.8 $\pm$ 40.3	105.2 $\pm$ 32.6 <sup>†</sup>
Cadmium	0.81 $\pm$ 0.41	1.45 $\pm$ 0.85 <sup>†</sup>	1.03 $\pm$ 0.39	1.57 $\pm$ 0.85 <sup>†</sup>

Results are expressed as mean  $\pm$  sd.

<sup>†</sup>P<0.05 vs pretreatment value by paired t-test.

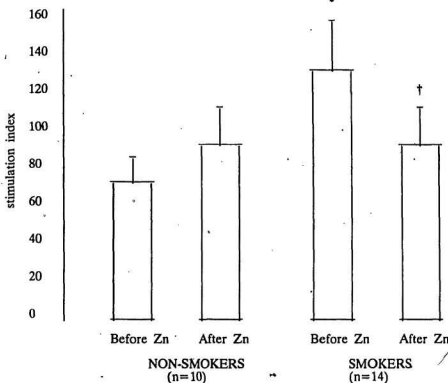
LYMPHOCYTE TRANSFORMATION RESPONSE TO  
PHYTOHAEMAGGLUTININ

FIG. 6.1

Lymphocyte transformation response to phytohaemagglutinin in non-smokers and smokers lymphocytes before and after zinc treatment. Results are expressed as stimulation index which represents the ratio between the mean counts per minute of triplicate cultures stimulated with mitogen divided by the mean counts per minute of triplicate control cultures. Bars represent mean  $\pm$  SD of each group. Smokers had higher ( $*P < 0.05$ ) stimulation index compared to non-smokers. Zinc treatment reduced ( $\dagger P < 0.05$ ) the response in smokers to non-smokers' level.



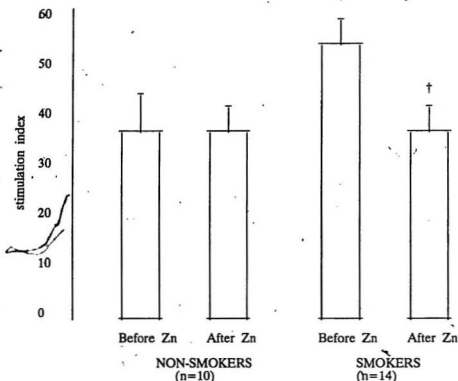
LYMPHOCYTE TRANSFORMATION RESPONSE TO  
CONCAVALIN A

FIG. 6.2

Lymphocyte transformation response to concanavalin A in non-smokers and smokers before and after zinc treatment. Results are expressed as stimulation index which represents the ratio between the counts per minute of triplicate cultures stimulated with mitogen divided by the mean counts per minute of triplicate control cultures. Bars represent mean  $\pm$  SD of each group. Smokers had higher (\* $P < 0.05$ ) stimulation index compared to non-smokers. Zinc treatment reduced ( $\dagger P < 0.05$ ) the response in smokers to non-smokers' level.

## NATURAL-KILLER CELL ACTIVITY

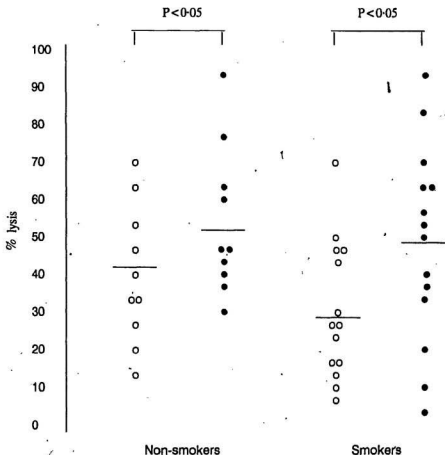


FIG. 6.3

Natural-killer cell activity (expressed as % lysis) in non-smokers and smokers before (o) and after (•) zinc treatment. Each point represents one value and the bars represent the mean value in each group. Smokers tended ( $P > 0.05$ ) to have low baseline natural-killer cell activity as compared to non-smokers. After zinc treatment the activity increased ( $P < 0.05$ ) in both non-smokers and smokers.

be required to delineate the possible mechanism for such difference between the mouse and human work. *In vitro* lymphocyte transformation response to PHA and Con A was increased in smokers. Silverman et al., (1975) also reported increased lymphocyte transformation response in young smokers. Zinc reduced the response to control values. The increased reactivity in these subjects may reflect their relatively young age and relatively shorter duration of exposure to cigarette smoke. Given a large exposure and older subjects smoking is associated with reduction in such lymphocyte responses (Silverman et al., 1975). Natural-killer cell activity, which is known to be suppressed in heavy smokers (Phillips et al., 1985), was seen to be slightly reduced in this study, however, zinc treatment increased the response in both smokers and non-smokers. Serum total cholesterol and LDL-cholesterol tended to be high while HDL-cholesterol tended to be low in smokers. Similar alterations in serum lipid profile in heavy smokers have been reported by others (Brischetto, Connor, Connor and Matarazzo, 1983; Stamford, Matter, Fell, Sady, Papanek and Cresanta, 1984). Zinc treatment had no marked effect on the serum lipid profile, LDL-cholesterol decreased slightly but significantly in smokers following zinc treatment. Following zinc supplementation, plasma zinc levels increased in both groups while copper levels decreased. Decrease in plasma copper by zinc has been reported by others (Brewer, Hill, Prasad, Cossak and Rabbani, 1983). Plasma cadmium level was slightly increased in smokers, and zinc treatment increased the level further in both groups. The cause of increased plasma cadmium by zinc is not known, it might represent an increased mobilization of cadmium from body stores by zinc.

The results show that a moderate dose of zinc can correct some smoking-induced alterations of the cellular immune functions in this study population. It is tempting to speculate that the beneficial effect of zinc on the immune alterations in smokers was the result of cadmium-zinc interaction, but further studies need to be done before a definite conclusion can be reached. Studies should be done to confirm if cadmium, indeed, is the cause of immune derangements seen in smokers. Although the effect of zinc on immunity among smokers may be a non-specific effect, this is unlikely, as the effect was only observed in smokers and not in non-smokers. That cadmium and zinc interaction do occur in relation to cigarette smoking was demonstrated in studies by Kuhnert, Kuhnert, Debanne and Williams (1987) and Kuhnert, Kuhnert, Erhard, Brashear, Groh-Wargo and Webster (1987). It was shown that in pregnant women who smoked, low birth weight of babies was correlated with increased cadmium level in the placenta and decreased zinc level in fetal circulation. Based on the results it was suggested that zinc supplementation should be considered as a partial means of intervention for the pregnant smokers.

It has historically been difficult to prove causality between smoking and disease. However, with time, enough evidence has accumulated that at present little doubt remains as to the adverse effects of smoking. Similarly, it is difficult to prove causality in the present study between cadmium and zinc variables and immune dysfunction. However, the results do suggest that an interaction may exist, and if it can be conclusively shown, then zinc treatment may prove to be a useful form of secondary

prevention of some ill-effects of cigarette smoking in those who fail to give up the habit.

## 6.9. SUMMARY AND CONCLUDING REMARKS

In this study it was demonstrated that cadmium at an oral dose of 50 ppm affects the number and function of suppressor T cells and natural-killer cells in mice. As a result IgM and IgG antibody production was increased following cadmium treatment and the natural-killer cell activity was reduced. Although cadmium is known to have a long half-life in tissues, its effects on the immune system in this study were short lived and the immune functions of mice recovered within 3 weeks of cessation of treatment. However, in the kidneys, which were also affected as evidenced by ultrastructural abnormalities, the effects of cadmium toxicity persisted even 6 weeks after cessation of treatment. The other important finding of this study was the demonstration that zinc at an oral dose of 500 ppm prevented the cadmium-induced immunopathology.

The mechanism(s) of cadmium-immunopathology and its prevention by zinc can be mediated through the mutual antagonism between the two metals for different structural and regulatory proteins. It is known that zinc is essential for the function of many metalloenzymes some of which are required for immune functions, and for the structural integrity of biomembranes, nucleic acids and gene expression regulatory proteins. It is possible that cadmium displaces zinc from these essential sites to exert its toxic effects and a large doses of zinc prevents this phenomenon. Cadmium can also interfere with zinc metabolism in tissues or cause a functional

zinc deficiency in the immune cells and zinc counteracts these effects. Further studies should be done to test these hypotheses.

Cadmium-induced immunopathology partially explains the observation of increased infection and malignancy in laboratory animals treated with cadmium. In humans, the possible immunotoxic potential of the metal is not greatly appreciated, but demonstration of immunotoxicity by a low dose of cadmium in this study suggests that cadmium may well be immunotoxic in man. Cadmium may thus be an additional risk factor for the development of certain diseases in man like atopic diseases, infection and malignancy, all of which are more common in people exposed to unusual concentrations of cadmium. Cigarette smokers are one such group who are known to acquire large amounts of cadmium from this source. Although the possible immunotoxic compound(s) in cigarette smoke is not known, cadmium is a likely candidate. Studies can be done to further characterize the immunological effects of cigarette smoking; role of cadmium, if any, in the phenomenon; and the effect of zinc on smoking induced immunopathology. Results of the preliminary study done as an extension of the mouse work, suggest that zinc may beneficially affect the immune system alterations seen in cigarette smokers. This finding may thus lay a fertile ground for future research.

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